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Thymic nuclear ADPRT

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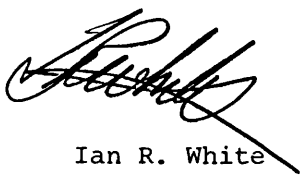

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To my parents

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ABSTRACT

In view of increasing concern with regard to the heterogenous nature of ADPRT inhibitor mediated effects *in vivo* an alternative means of specific enzyme inhibition was sought.

The proposed route of the study was to use polyclonal antiserum raised against purified enzyme to isolate ADPRT specific mRNA species *via* polysome precipitation. Such species were then to be used for synthesis of cDNA sequences which when incorporated into inducible vectors would generate antisense mRNA species in transfected cell lines.

The aims of the project were partly realised.

A three-step protocol was developed for purification of the enzyme from porcine thymus, comprising extraction of PEI treated nuclei, HA and Sepharose-3AB affinity chromatography. Typically 5000 fold purification was achieved, although minor contamination proved problematic, necessitating preparative gel electrophoresis as a final clean up step.

The enzyme was observed to be a single polypeptide of approximately 116 Kd by SDS-PAGE under reducing conditions, basic (P_i ~9.5 to 10) and to have a blocked NH_2-terminus.

Attempts were made to obtain protein sequence data by purification and analysis of cyanogen bromide cleavage products, but were hindered by unsuccessful resolution of fragments by reverse phase HPLC.

Antiserum was successfully raised against the enzyme in rabbits, using agarose/ADPRT gel pieces as immunogen, and characterised by double diffusion in agarose, immune inhibition of enzyme activity, ELISA and Western blotting.

ABBREVIATIONS

ADPR	adenosine diphosphoribose
ADPRT	nuclear ADP-ribosyl transferase
3AAB	3-acetylaminobenzamide
3AB	3-aminobenzamide
3ABA	3-aminobenzoic acid
BA	benzamide
DMF	dimethyl formamide
DMS	dimethyl sulphate
DMSO	dimethyl sulphoxide
DTT	dithiothreitol
EDAC	1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide
ELISA	enzyme linked immunosorbant assay
HA	hydroxyapatite
3HB	3-hydroxybenzamide
HPLC	high performance liquid chromatography
HRPO	horse radish peroxidase
IEF	isoelectric focussing
i.m.	intramuscular
Kd	kilodaltons
β-ME	β-mercaptoethanol
3MeB	3-methoxybenzamide
mol. wt.	molecular weight
NEM	N-ethylmaleimide
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEI	polyethyleneimine
P_i	isoelectric point
PRAMP	2'-(5"-phosphoribosyl)-adenosine-5'-monophosphate

ABBREVIATIONS (continued)

RIA	radioimmunoassay
R-5-P	ribose-5-phosphate
3SB	3-succinimidyl benzamide
3SBA	3-succinimidyl benzoic acid
s.c.	subcutaneous
SDS	sodium dodecyl sulphate
SPB	standard phosphate buffer
SVPDE	snake venom phosphodiesterase
TCA	trichloroacetic acid
TEA	triethanolamine
TEMED	N,N,N',N'-tetramethylethylenediamine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
u.v.	ultraviolet

CONTENTS

CONTENTS (continued)

CHAPTER 1

General Introduction

1.1 Introduction

Enzymic ADP-ribosylation of proteins is widely recognised as one of the most commonly occurring post translational modification systems in cellular metabolism.

Two distinct species of enzyme are responsible for such reactions, *i.e.*, mono ADP-ribosyl transferases, found in both prokaryotic and higher organisms, and ADP-ribose polymer synthesising ADP-ribosyl transferases found only in the nuclei of eukaryotic organisms.

1.2 Mono ADP-ribosyl transferases

The enzymes comprising this group cleave the high energy nicotinamide-ribose bond of oxidised NAD and covalently attach the liberated adenosine diphosphoribose (ADPR) residue to defined receptor species *via* N-glycosidic linkages. The pathological effects of Bordella pertussis toxin, cholera toxin and *E. coli* enterotoxin in mammals are now known to be due to disruption of adenylate cyclase metabolism through mono ADP-ribosylation of guanine nucleotide binding proteins, whereas the modification of elongation factor 2 by Diptheria toxin and *Pseudomonas aeruginosa* toxin A directly inhibits protein synthesis. A number of endogenous vertebrate mono ADP-ribosyl transferases have also been shown to exist (for reviews see Vaughan and Moss, 1981; 1985 and Ueda and Hayaishi, 1985).

1.3 Poly ADP-ribosylation

The work presented and discussed in this thesis primarily concerns poly ADP-ribosylation by the nuclear ADP-ribosyl transferase enzyme (ADPRT) The enzyme differs from mono ADP-ribosyl transferases fundamentally in

that the initial reaction leads to an O-glycosidic linkage and that the ADPR-protein adduct thus formed can subsequently serve as a receptor for further modification, that is ADPR chain formation by the sequential addition of further ADPR residues linked in an α(1" → 2') glycosidic fashion.

The enzyme responsible for the process, which was first observed in Mandel's laboratory over twenty years ago (Chambon *et al.*, 1966) is tightly associated with chromatin. Despite, however, extensive studies to determine the function(s) of ADPR polymer formation, the biological role of the process has yet to be elucidated.

The field of poly ADP-ribosylation is currently the subject of investigation by many groups of researchers and to review comprehensively all of the published literature would be a huge task. The following survey is therefore intended only to present some of the areas of interest and to highlight a number of inherent problems associated with studying this particularly intriguing process.

A number of workers have recently reviewed the field (Gaal and Pearson, 1985; Ueda and Hayaishi, 1985).

1.3.1 ADPRT

ADPRT has been found to be a ubiquitous enzyme in higher eukaryotes with particularly pronounced specific activity in lymphatic tissue (Agemori *et al.*, 1982).

The results of a number of published purification protocols are presented in Table 1 and with the exception of Tsopanakis and co-workers (1978), who used aqueous/organic solvent systems at sub-zero temperatures, most of the methodology combines reliable established techniques such as ion exchange, gel filtration, hydroxyapatite, and

TABLE 1: Literature Purification Protocols Summarised

Source	No. of steps	Purification (fold)	Molecular weight (Kd) SDS PAGE	Molecular weight (Kd) Gel filtration	K_m (μm)	P_i	Yield (%)	Reference
Calf thymus	8	3111	120	120	82	6.5	6	Mandel *et al.*, (1977)
Calf thymus	6	1333	130	150-60	60	-	17	Yoshihara *et al.*, (1978)
Calf thymus	4	1250	120	115	55	9.8	14	Ito *et al.*, (1979)
Calf thymus	5	1490	114	-	-	-	14	Zahradka and Ebisuzaki (1984)
Calf thymus	6	540	-	-	100	-	3.4	Okazaki *et al.*, (1976)
Lamb thymus	6	2900	135	175	160	9.6	5	Petzold *et al.*, (1981)
Pig thymus	5	9200	63.5	58	23	8.4	47	Tsopanakis *et al.*, (1978)
Sheep testis	4	740	120	-	57	-	24	Zhang and Qiu (1986)
Rat liver	7	5300	-	-	25	-	15	Okayama *et al.*, (1977)
HeLa	5	2200	112	150	46	-	24	Jump and Smulson (1980)
Mouse testis	3	864	116	-	47	-	11	Agemori *et al.*, (1982)
Human placenta	6	1924	116	-	61.7	10	17	Ushiro *et al.*, (1987)
Human placenta	4	4900	115	-	52	-	7	Burtscher *et al.*, (1986)
Erlich ascites tumour	4	698	130	-	20	9.4	21	Kristensen and Holtlund (1978)

in a few cases, affinity chromatography (Okazaki *et al.*, 1976; Burtscher *et al.*, 1986; Ushiro *et al.*, 1987).

The enzyme has been shown to be a single polypeptide of 110 to 120 Kd by SDS-PAGE analysis under reducing conditions. A few higher estimations have been published, but have usually resulted from assuming the molecular weight of β-galactosidase, a commonly used molecular weight marker, to be 130 Kd, rather than 116 Kd. The size of the pig thymus enzyme recorded by Tsopanakis and co-workers (1978) probably resulted from proteolysis of the native enzyme during the latter stages of purification. In support of the majority of size estimations, Scovassi and co-workers (1984, 1986) have developed a technique coupling SDS-PAGE with *in situ* renaturation of the separated proteins. Incubation of the intact gel with [^{32}P] NAD^+, followed by autoradiography of the dried gel, allows visualisation of the enzymically active protein bands. Using this technique, they found that vertebrates, arthropods and molluscs all had catalytic peptides of 115 to 120 Kd (Scovassi *et al.*, 1986).

Gel filtration analyses of molecular weights have tended to be larger than electrophoretic determinations, but are probably due to asymmetry of the polypeptide. Frictional ratios of 1.39 and 1.81 have been recorded for the calf enzyme (Ito *et al.*, 1979; Ohgushi *et al.*, 1980) and 1.82 for the human placental enzyme (Ushiro *et al.*, 1987).

The NH_2 terminus has been found to be blocked in pig (Tsopanakis *et al.*, 1978), calf (Ito *et al.*, 1979) and Erlich ascites tumour (Holtlund *et al.*, 1980) enzyme and subsequently no direct NH_2 terminal protein sequence data has been reported.

Total amino acid analysis has shown only slight species specific variation (Agemori *et al.*, 1982; Ito *et al.*, 1979; Holtlund *et al.*, (1981). The enzyme is basic, with high lysine rather than arginine content, and has an isoelectric point (P_i) of approximately pH 10. Lower P_i values probably reflect the result of DNA enzyme complexes (Tsopanakis *et al.*, 1978; Mandel *et al.*, 1977).

The purified enzyme absolutely requires DNA for activity (Yoshihara *et al.*, 1978; Benjamin and Gill 1980a) and it has been shown by Yoshihara and co-workers (1978) and other groups, that DNA closely associated with the enzyme, termed active DNA, is a far more efficient activator than total calf thymus DNA. Half maximal activation of one enzyme molecule by active DNA was estimated to occur with an average of 10 base pair fragments, compared to 220 to 240 base pairs for total DNA. Similar figures of 8 and 320 base pair fragments were also recorded by Niedergang and co-workers (Niedergang *et al.*, 1979). A specific binding site was thus suggested.

Zahradka and Ebisuzaki (1984) found the enzyme to be a zinc containing metalloprotein and implicated the zinc containing site as DNA binding. They suggested that the G2 cell cycle arrest of Euglena cells, recorded by Falchuk and co-workers (Falchuk *et al.*, 1975) following zinc depletion, may be connected with alterations in ADPR metabolism.

With the exception of the pig thymus enzyme purified by Tsopanakis and co-workers (1978), histones are not absolutely required for enzyme activity. The presence of histones, and histone H1 in particular, do, however, stimulate catalysis. Ito and co-workers (1979) suggested that histone H1 caused allosteric stimulation of ADPRT following

observations of a two-fold increase in polymer formation in its presence. Petzold and co-workers (1981) observed a ten-fold increase in activity of lamb thymus enzyme in the presence of histone H1, in agreement with similar observations by Okayama and co-workers (1977) for rat liver enzyme. Both groups found high levels of histone to be inhibitory. A decrease in the K_m of the enzyme, coupled with increased v_{max} values usually accompany histone addition (Kristensen and Holtlund, 1978; Okayama *et al.*, 1977).

The requirement of the enzyme for Mg^{2+} ions and thiol compounds is also well documented.

In the absence of $MgCl_2$, a reduction of 20% in enzyme activity was recorded by Ushiro and co-workers (1987) for human placental ADPRT, whereas reductions of 53% and 85% were recorded for calf thymus enzyme activity by Ito and co-workers (1979) and Yoshihara and co-workers (1978) respectively. Yoshihara and co-workers also observed that Mg^{2+} ions enhanced automodification of the enzyme through increased chain number, as well as increasing chain length and number on modified histone H1. Mg^{2+} depletion has recently been promoted as an experimental means of enhancing modification of exogenously added receptors *in vitro* (Tanaka *et al.*, 1985). Exceptionally, Mg^{2+} insensitive systems have been recorded (Benjamin and Gill, 1980b; Petzold *et al.*, 1981).

In the absence of dithiothreitol (DTT) or β-mercaptoethanol (β-ME) or in the presence of sulphydryl binding agents such as N-ethylmaleimide (NEM) or *p*-hydroxymercuribenzoate (PHMB), polymer formation is reduced.

Omission of DTT has been recorded to inhibit activity by as much as 39% (Ito *et al.*, 1979) and 43% (Jump and Smulson 1980), whereas 1 mm

NEM inhibited calf thymus enzyme by 94% (Ohgushi *et al.*, 1980) and lamb thymus enzyme by 50% (Petzold *et al.*, 1981).

The locations of the DNA and NAD^+ binding sites and the automodification site have been investigated by Shizuta's group (Shizuta *et al.*, 1987).

Limited digestion of the purified calf thymus enzyme with papain yields fragments of 74 and 46 Kd. The 46 Kd fragment contains the DNA binding domain and the 74 Kd fragment the automodification domain. Upon prolonged digestion of ADPRT, modified with [^{3}H]-ADPR, all of the radioactivity was found to be associated with a 23 Kd fragment (Nishikimi *et al.*, 1982). The structure was further clarified by α-chymotrypsin digestion of the enzyme, which yielded two fragments of 54 Kd and 66 Kd. The smaller fragment contained the NAD^+ binding site, and following papain digestion, the other fragment could be resolved into a DNA binding domain (46 Kd) and an automodification domain (22 Kd) (Kameshita *et al.*, 1984).

Incubation of the purified 74 and 46 Kd fragments from α-chymotrypsin digestion in the presence of DNA resulted in recovery of 20% of the native enzyme activity (Kameshita *et al.*, 1986). Furthermore, the large fragments obtained from either papain or chymotrypsin digestion were more efficient receptors than the 22 Kd fragment alone, when incubated in the presence of native polymerase.

Broad species cross reactivity of polyclonal antiserum raised against calf thymus enzyme has been observed. Ikai and Ueda (1980) found that rat, mouse, chicken and HeLa cell enzyme activity could

be immuno-precipitated by antiserum raised in rabbits, whereas Jongstra-Bilen and co-workers (1981) found similarly sized immuno-reactive peptides when probing blots of calf, rat, chicken and pig enzyme. More recently Scovassi and co-workers (1986) identified major cross reactive peptides of 100 to 120 Kd in HeLa cells, calf thymus and chick embryo and less pronounced reactive peptides in reptiles and fishes. A number of smaller bands were also detected, suggesting extensive proteolytic degradation. Indeed, when extractions of pig tissue were prolonged, Petzold and co-workers (1981) observed that an extra band became increasingly apparent at 57 Kd, which could correspond to the molecular weight recorded by Tsopanakis and co-workers (1978). 44 Kd and 40 Kd fragments were found to be the only immuno-reactive peptides in spinach and yeast extracts respectively (Kameshita *et al.*, 1985), although whether they were degradation products of larger polypeptides was unclear. In general then, the structure/function relationship of the enzyme seems to have been preserved throughout evolution, in common with that found for a number of other DNA bound proteins.

Monoclonal antibodies have been generated specifically for the NAD^+ binding site (Lamarre *et al.*, 1986) and the DNA binding site (Kameshita *et al.*, 1985; Lamarre *et al.*, 1986). Their use as probes for ADP-ribosylation reactions has been suggested in permeabilised cell and microinjection systems, although DNA binding domain epitopes may prove to be shielded in such environments.

Observation of differential binding sites of the protein in chromatin have also been recorded. Giri and co-workers (1978) found that the enzyme activity was located primarily in the linker region

between successive nucleosomes. Digestion of dinucleosomes with micrococcal nuclease was found to result in enzyme activity being associated only with the monomer bound to the linker. Furthermore, isolation of enzymically active domains using immobilised anti ADPR antibodies, selectively to bind modified regions of the chromatin, showed that enzyme activity could be correlated with domains containing increased strand breaks (Malik *et al.*, 1983). In contrast, Leduc and co-workers (1986), using indirect colloidal gold labelling combined with electron microscopy studies, found that the calf enzyme was primarily located bound to the nucleosomal core.

1.3.2 $(ADPR)_n$ structure and characterisation

Chromatographic and enzymic analysis have been used predominantly to study $(ADPR)_n$ structure.

Hydroxyapatite chromatography was shown to be a reliable means of separating ADPR polymer from nucleic aids (Sugimura *et al.*, 1971) and resolving mixed oligomeric ADPR species (Tanaka *et al.*, 1977). DEAE-cellulose has similarly been used to separate oligomers up to 11 residues long (Kawaichi *et al.*, 1981a), while gel filtration combined with electrophoresis has been successfully used to resolve polymeric ADPR up to 70 residues (Hayashi *et al.*, 1983).

The polymer is stable in alkali (Sugimura, 1973) and has been suggested to have a helical conformation (Minaga and Kun, 1985). It is resistant to DNase, RNase and micrococcal nuclease, but susceptible to cleavage by snake venom phosphodiesterase (SVPDE) and poly ADPR glycohydrolase (Miwa *et al.*, 1974) (see Figure 1). Labelling of ADPR pools with radioactive NAD^+, followed by analysis of such enzymic digestion products, has been used widely to gain more specific information regarding polymer structure and size.

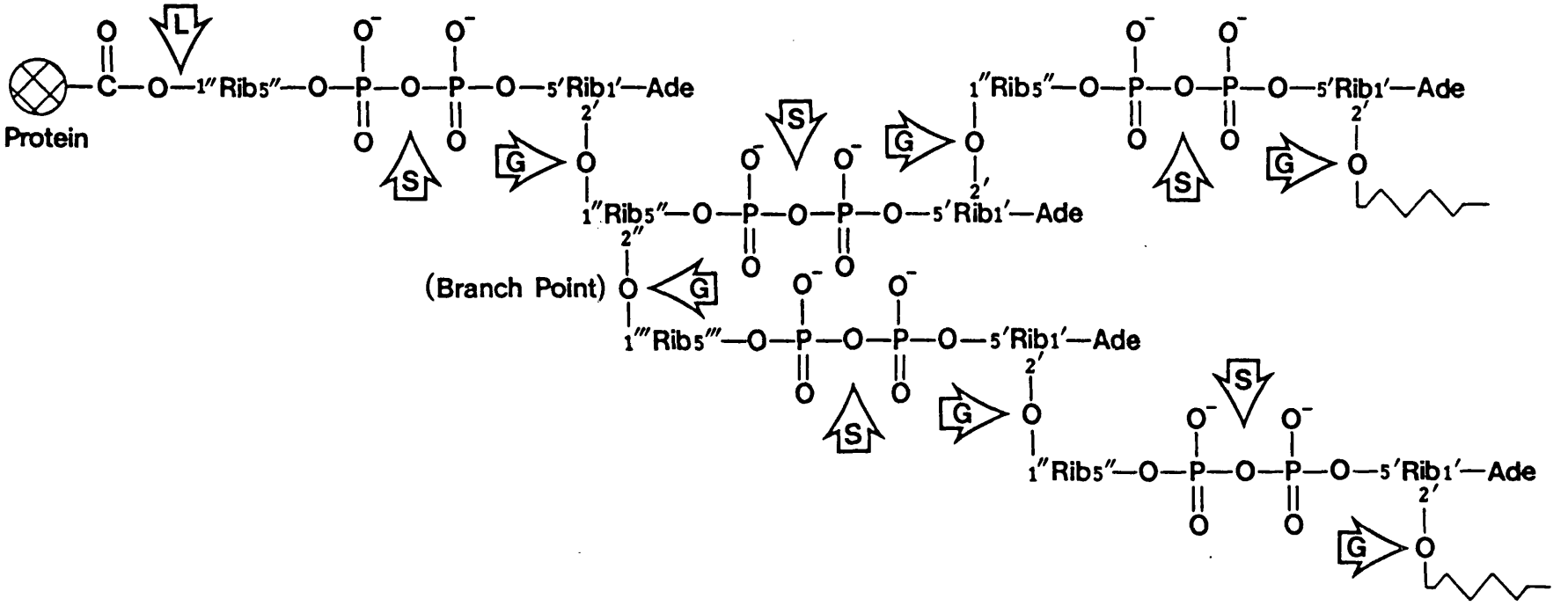


Figure 1 Structure of ADPR polymer. The sites of cleavage by ADP-ribosyl protein lyase (L), snake venom phosphodiesterase (S), and poly ADPR glycohydrolase (G) are indicated by arrows.

For each linear polymer, the enzyme SVPDE releases one molecule of 5'-AMP and one molecule of 2'-(5"-phosphoribosyl)-5'-AMP(PRAMP), a molecule unique to poly ADPR, for every internal ADPR residue. The two species may be separated by paper or thin layer chromatography, although HPLC is increasingly being used, and quantified. The average chain length may then be calculated by the equation:

$$\text{Average chain length} = \frac{[5'\text{-AMP}]+[\text{PRAMP}]}{[5'\text{-AMP}]}$$

(Nishizuka *et al.*, 1969)

Using this ratio, the majority of size estimations did not, however, exceed 30 or so residues per chain. This apparent limitation was subsequently explained by the observations of Miwa and co-workers (1979). Using HA chromatography, followed by DEAE-Sephadex A-25 chromatography of SVPDE digested polymer, they observed that a novel compound, comprising 2% of the total polymer, eluted after the PRAMP peak. This was identified as 2'-[1"-ribosyl-2"-(1"'-ribosyl)]adenosine-5',5",5"'-tris(phosphate) (PR_2AMP) and a branched polymer structure was postulated. This molecule was also isolated by Hayashi and co-workers (1983) and the observations were further supported by electron microscope studies on calf thymus ADPR polymer. An average chain length of 30 residues was calculated enzymatically, although electrophoresis clearly resolved ADPR species much larger in size. Electron microscopy showed a branching structure to the polymer and modification of up to 350 residues per chain was calculated, with branching occurring on average every 35 residues.

Calculations for polymer size estimations therefore had to be revised (Miwa and Sugimura, 1982):

$$\text{Average polymer size} = \frac{[5'\text{-AMP}] + [\text{PRAMP}] + [(\text{PR})_2\text{AMP}]}{[5'\text{-AMP}] - [(\text{PR})_2\text{AMP}]}$$

and

$$\text{Average branches per molecule} = \frac{[(\text{PR})_2\text{AMP}]}{[5'\text{-AMP}] - [(\text{PR})_2\text{AMP}]}$$

The inadequacies of not allowing for branching were also well illustrated recently by Alvarez-Gonzalez and Jacobson (1987). In nucleotide permeable mouse cells, polymers up to 190 residues were formed with up to 5 branches, whereas not allowing for branching, the average chain length calculated for the largest polymers was 28. Polyacrylamide gel electrophoresis studies also gave excellent agreement with size estimations.

Three means of attachment of polymer to nuclear receptor species predominate. The major linkage is *via* a carboxylic acid ester bond and is characterised classically by its sensitivity to cleavage by neutral NH_2OH (Adamietz and Hilz, 1976). The remaining bonds, comprising 30% of the total rat liver ADPRT bound polymers, exist as 15% alkali sensitive and 15% alkali insensitive (Kawaichi *et al.*, 1981b). The former probably result from modification by a nuclear mono ADPRT (Tanigawa *et al.*, 1984) and the latter from non-enzymic, chemical modification, as shown by Hilz and co-workers (Hilz *et al.*, 1984). Indeed, Tanigawa and co-workers (1984) were able to show that mono ADPRT synthesised ADPR-protein species were efficient receptors for the polymerase and it seems probable that chemically synthesised adducts will also serve as receptors, assuming that no site specific hindrance occurs.

1.3.3 $(ADPR)_n$ quantitation

Quantitation of $(ADPR)_n$ *in vivo* and *in vitro* poses a number of difficult problems. The lack of a specific precursor enabling labelling of ADPR pools to high specific activity, the rapid turnover of ADPR residues and the low endogenous levels of ADPR have therefore provoked a number of varied approaches.

Stone and co-workers (1976) used isotope dilution to quantify ADPR levels, although this proved difficult with some tissues, as well as being laborious, requiring purification of the ADPR pool to constant specific activity.

Various immuno-quantitation methods have been reported utilising antibodies raised against 5'-AMP, PRAMP and ADPR polymer.

Bredehorst and co-workers (1978) used alkali treatment to cleave mono ADPR residues to 5'-AMP and R-5-P and then used highly specific antibodies raised against 5'-AMP to quantify ADPR by radioimmunoassay (RIA) in the order of 1-40 pmol. Boronate affinity chromatography was used to supplement further RIA in an effort to reduce contamination by nucleic acid degradation (see section 1.3.6) (Wielckens *et al.*, 1981). Following enzymic degradation of the polymer, antibodies raised against 5'-AMP and PRAMP were used to determine a polymeric ADPR level of 85 pmol per gram of rat liver tissue using RIA. Furthermore, mono ADPR residues were found to be in 200-fold excess over polymeric residues.

However, levels recorded by the above method were prone to underestimation, due to incomplete binding to the boronate column and incomplete enzymic digestion. Adamietz and Bredehorst (1981) used alkali in the presence of Mg^{2+} ions to degrade ADPR polymer directly

to PRAMP and 5'-AMP and suggested that such a cleavage method would allow determination of chain lengths *in vivo*.

Antibodies raised against the polymer have been successfully used to detect nanogram levels of polymeric ADPR by RIA. The method is of limited use, however, as antibody/antigen interactions proved to be chain length dependant (Sakura *et al.*, 1977).

By far the most sensitive and reliable quantitation methods to date have involved fluorescence detection of derivatised nucleotides.

Niedergang and co-workers (1978) used enzymic hydrolysis of the polymer, followed by derivatisation and measurement by the method of Yuki and co-workers (1972). Unfortunately, the method measures all adenine nucleotides and is therefore critically dependant on removal of contaminating nucleic acid species.

A more sensitive and selective technique has since been developed, which is capable of detecting 1 pmole of ADPR proving 30 times more sensitive than the above method (Sims *et al.*, 1980; Juarez-Salinas *et al.*, 1983). The unique nucleotides ribosyl adenosine and di-ribosyl adenosine are released from linear and branched ADPR polymers by digestion with SVPDE and phosphomonoesterase. The fluorescent ethenoderivatives are then separated by HPLC and measured.

1.3.4 $(ADPR)_n$ degradation

While the polymerisation reaction has received the vast majority of attention over the past twenty years, the turnover of ADPR polymer has been sadly neglected. A major reason for this dearth of activity has been the unavailability of a specific means of modulating polymer

breakdown, *i.e.*, specific inhibition. Had such complete control of ADPR metabolism been possible, the elucidation of its biological function may have been significantly accelerated. Breakdown of poly ADPR is mediated by the action of three enzymes (Figure 1).

Poly ADPR glycohydrolase is by far the most active enzyme and was first observed by Miwa and Sugimura (1971). It has been purified to homogeneity by a number of groups and shown to be a monomeric protein of approximately 59 Kd in size with a pH optimum of 7.5 (Tavassoli *et al.*, 1983; Hatakeyama *et al.*, 1986; Tanuma *et al.*, 1986a). It is stimulated by thiolated compounds such as DTT and β-ME and inhibited by ADPR, cyclic AMP, histones, polyamines and single stranded DNA (Tavassoli *et al.*, 1983; Hatakeyama *et al.*, (1986).

The enzyme has been shown to be predominantly exoglycosidic in action, sequentially removing single ADPR residues from the free adenosine termini of the polymer and is capable of degrading branched, as well as linear, polymers. Hatakeyama and co-workers (1986) observed that the K_m of the enzyme for small polymers was 2 orders of magnitude higher than the K_m for large polymers (>20 residues) and that degradation could accordingly be separated into a fast, processive stage, and a slower, non-processive stage. Such a process explained the earlier observation of Lorimer and co-workers (1977) that chain length appeared to be inversely proportional to glycohydrolase activity. Hatakeyama and co-workers therefore suggested that glycohydrolase may exert cellular mediation of ADP-ribosylation at two distinct levels.

Endogenous phosphodiesterases could play an active part in polymer metabolism, although the K_m for the polymer has been reported to be 1 to 2 orders of magnitude higher than that of glycohydrolase (Miwa and Sugimura, 1982), suggesting a minor pathway of degradation. The probably insignificant role of phosphodiesterase is further emphasised by the failure to detect significant levels of PRAMP, the product of phosphodiesterase action, in *in vivo* studies.

The final, rate limiting, constituent of polymer degradation is ADP-ribosyl protein lyase. The protein has been isolated from rat liver cytoplasm and is approximately 80 Kd in size (Oka *et al.*, 1984). It specifically cleaves mono ADPR-carboxylate esters, the importance of which may be critical in terms of the vast predominance of mono ADPR residues *in vivo*, relative to oligo and poly ADPR. It has been suggested that defects in ADP-ribosyl protein lyase activity may be responsible for Glutamyl ribose-5-phosphate storage disease, a possible rare clinical manifestation resulting from faulty ADP-ribosylation metabolism (Williams *et al.*, 1984).

1.3.5 Inhibition of ADPRT

Much of the evidence supporting the widespread involvement of ADP-ribosylation in eukaryotic chromatin metabolism has resulted from extensive enzyme inhibition studies. The inhibitors utilised may be broadly divided into four main categories: nicotinamide, a product of the enzymic reaction (Sugimura, 1973); thymidine (Sugimura, 1973); methylated xanthines, such as caffeine and theophylline (Levi *et al.*, 1978); and benzamide and its 3-substituted derivatives (Purnell and Whish, 1980).

The 3-substituted benzamides developed at Bath have been exhaustively used by many workers due largely to their high potency [3-aminobenzamide (3AB) and 3-methoxybenzamide (3MeB) have K_i values of 2.6 and 0.6 μM respectively for the pig thymus enzyme (Purnell, 1980)] and what was thought to be their high specificity.

Their use as probes for ADP-ribosylation reactions and hence results obtained from their use, have come under increasing criticism, however, with regard to the latter point.

Cleaver's group in particular have examined non specific effects associated with their use and suggested that they inhibit *de novo* purine synthesis, an effect which could lead to major perturbations in repair synthesis and strand break metabolism (Cleaver *et al.*, 1983).

In support, it was argued that cell lines deficient in nucleotide salvage pathways would be significantly more sensitive to 3AB toxicity (Cleaver, 1984). This was borne out by the observation that cell survival was reduced by 60%, relative to wild type cells, in a mutant cell line which was deficient in hypoxanthine guanine phosphoribosyl transferase, a critical enzyme in nucleotide salvage pathways. In a more extensive study, it was observed that 3AB above 5 mM caused significant inhibition of cell growth and that at such levels the inhibitor enhanced deoxycytidine incorporation into DNA. In direct contrast, incorporation of deoxyadenosine, deoxyguanosine and thymidine was slightly depressed (Milam *et al.*, 1986). More significantly, at 1 mM, 3AB reduced incorporation of the methyl group of [^{3}H]methionine into deoxyguanosine and deoxyadenosine by 50% and deoxycytosine by 90%. These results were consistent with the

interpretation of Hunting and co-workers (1985) that folate metabolism, and not *de novo* synthesis was the probable target of the inhibitors. Hunting's group had found that of the inhibitors 3AB , 3-acetylaminobenzamide (3AAB) and 3MeB, only 3MeB inhibited conversion of hypoxanthine to ATP and GTP, whereas the incorporation of radioactive formate and glycine was reduced by all three.

Competitive inhibition of deoxyglucose uptake for a number of cell lines has also been recorded (Grunfeld and Shigenaga, 1984), although the concentrations required to effect any significant alterations, however, are vastly in excess of those usually employed in physiological studies.

It seems possible, therefore, that a significant proportion of the results presented in this field to date are artifactual in nature, reflecting not just a monospecific ADPRT associated effect, but the net consequences of a variety of specific and non specific metabolic effects. Indeed, Cleaver and Morgan (1987) in a recent report have proposed that physiologically acceptable concentrations of inhibitor actually accelerate DNA excision repair (see section 1.4.1) and that the plethora of accounts to the contrary have resulted from using the inhibitors at unacceptably toxic levels.

1.3.6 $(ADPR)_n$ receptors

A well established approach designed to gain a greater understanding of nuclear ADP-ribosylation reactions has been to study the nature and variety of ADPR receptor species. In view of the lack of specific ADPR precursors and the vast heterogeneity of receptor molecule this has proved a difficult and challenging task.

However, the highly specific interaction of *cis*-diol compounds with free boronate residues has elevated boronate affinity chromatography to the foremost choice of technique for isolation and characterisation of such conjugates (Okayama *et al.*, 1978; Adamietz *et al.*, 1979).

ADPR receptors may be broadly divided into two main categories: histones and non-histones.

The histones have received much attention, both *in vitro* and *in vivo* (Bredehorst *et al.*, 1978; Okayama *et al.*, 1978; Poirier *et al.*, 1982; Jump and Smulson, 1980) with histone H1 being the major receptor in many cases. Interpretations of *in vitro* results are made difficult, however, as ADP-ribosylation patterns may vary considerably, due to increased nuclease digestion of DNA, during nuclei isolation, for example (Prentice and Gurley, 1983).

Poly ADP-ribosylation of histone H1 has been postulated to have a major role in modulation of chromatin structure, with particular regard to accessibility of DNA associated enzymes. Poirier's group have extensively studied histone H1 modification (Poirier *et al.*, 1982). They observed that modification of rat pancreatic poly-nucleosomes by purified calf thymus ADPRT caused a general relaxation of chromatin structure, identifying histone H1 as the major ADPR receptor. This was in agreement with the earlier work of Burzio and co-workers (1980), who demonstrated that histone H1 modification decreased histone-nucleic acid interactions, causing chromatin relaxation. DeMurcia and co-workers (1986) later found that this effect was reversible upon degradation of the polymer with ADPR glycohydrolase. Mandel's group added weight to the argument that enzyme accessibility was enhanced by observing increased DNA poly-

merase α activity in the presence of poly ADP-ribosylated polynucleosomes (Niedergang *et al.*, 1985). Hyper ADP-ribosylation of histone H1 has not been observed *in vivo* to date, however. Indeed, mono and oligo ADP-ribosylation seem to predominate (Ogata *et al.*, 1980; Adamietz, 1978), although this could be explained by fast turnover of polymer (Jacobson *et al.*, 1983; Wielckens *et al.*, 1982, 1983).

Of the non histone receptor species, many DNA associated enzymes have been shown to have their activities modulated *in vitro* by ADP-ribosylation. To date, DNA topoisomerase I (Ferro and Olivera, 1984; Jongstra-Bilen *et al.*, 1983), DNA polymerase α (Yoshihara, *et al.*, 1985), DNA polymerase β (Yoshihara *et al.*, 1985; Ohashi *et al.*, 1986), Seminal and pancreatic ribonuclease (Leone *et al.*, 1986; Suzuki *et al.*, 1986) terminal deoxyribonucleotidyl transferase (Yoshihara *et al.*, 1985; Tanaka *et al.*, 1986) and DNA ligase II (Yoshihara *et al.*, 1985) have all been shown to be inhibited by ADP-ribosylation. Inhibition of topoisomerase and ribonuclease was shown to be reversible on treatment of the modified enzymes with mild base (Ferro *et al.*, 1983; Leone *et al.*, 1986) and recently Ohashi (1986) showed that polymer size is related to the degree of inhibition of some enzymes *in vitro*.

Creissen and Shall (1982) have recorded the only example of enzyme activation after ADP-ribosylation and have postulated ADP-ribosylation of DNA ligase II to be a central feature in some DNA repair mechanisms (see later).

It seems likely that the attachment of negatively charged ADPR moieties to the enzymes may bring about reduced enzyme activity by

means of electrostatic repulsion from the negatively charged DNA backbone, thereby reducing accessibility (Ferro and Olivera, 1984; Tanaka *et al.*, 1984) as free ADPR polymer does not cause inhibition.

Probably the most unexpected, most interesting receptor in the non histone class is the enzyme ADPRT itself.

1.3.7 ADPRT automodification and regulation

The unusual enzymological feature of automodification has been demonstrated by many workers *in vitro* (Yoshihara *et al.*, 1977; Kristensen and Holtlund, 1976; Kawaichi *et al.*, 1981b; Zahradka and Ebisuzaki, 1982), in isolated nuclei (Jump and Smulson, 1980; Ogata *et al.*, 1981) and in permeabilised cells (Benjamin and Gill, 1980a).

The role of automodification, either as a chromatin structure regulation mechanism (Kawaichi *et al.*, 1981b) or as an intermediate in the modification of other receptor species (Yoshihara *et al.*, 1975) has received much attention.

Whether automodification is an intra- or inter-molecular phenomenon (Yoshihara *et al.*, 1977) has not been clearly demonstrated, although in an *in vitro* system, Holtlund and co-workers (1983) showed multisite modification of two enzymically inactive fragments by purified active calf thymus enzyme. An intermolecular mechanism was therefore favoured. Multisite modification was also observed by Kawaichi and co-workers (1981b), who showed that enzyme bound polymer was not transferred to other endogenous receptors *in vitro*, although the modified enzyme retained the ability to transfer mono ADPR residues from NAD^+ to histones. Inhibition of enzyme activity was observed with modification and an autoregulatory role was suggested.

Autoregulation was also proposed by Zahradka and Ebisuzaki (1982), who observed a reduction in the affinity of modified ADPRT for DNA, supporting the previous observations of Yoshihara and co-workers (1981). A restoration of affinity for DNA was observed, however, upon digestion of polymer with a semi-purified glycohydrolase preparation. A shuttling mechanism was suggested, whereby the enzyme could be repelled from DNA by automodification and re-associated by glycohydrolase mediated polymer degradation. The effects of stimulation of polymerase activity by histone or Mg^{2+} ions could therefore be explained by reduction of charge repulsion effects arising from negatively charged ADPR and DNA polymer species.

Two isolated observations of free polymer *in vitro* have been recorded (Rickwood *et al.*, 1977; Jump and Smulson, 1980) suggesting transfer of ADPR polymer from the automodified enzyme, but this has been largely interpreted to be the result of non enzymic cleavage of the ADPR-receptor linkage.

Automodification has recently been investigated by Taniguchi (1987) and been shown to occur by successive elongation *via* addition to the terminal AMP residue, in a 'tail out', rather than 'head out', mechanism.

Holtlund and co-workers (1983) suggested proteolysis of the enzyme as a means of control of ADP-ribosylation, following consistent observations of 59 Kd and 76 Kd fragments when purifying calf enzyme.

1.4 Cellular Functions of Poly ADP-ribosylation

A vast quantity of data is now available suggesting involvement of poly ADP-ribosylation in a whole host of processes fundamental to DNA metabolism.

However, while such widespread involvement is acknowledged, for reasons of brevity, only DNA repair, cell differentiation and proliferation, and the cell cycle, are discussed below.

1.4.1 DNA repair

The requirement of DNA for enzyme activity (Yamada *et al.*, 1971; Petzold *et al.*, 1981) and in particular, DNA containing strand breaks (Benjamin and Gill, 1980a; Ohgushi *et al.*, 1980) has strongly implicated ADPR metabolism in DNA repair processes.

Benjamin and Gill (1980a,b) showed that while undamaged DNA was not an effective activator of the polymerase, damaged DNA stimulated enzyme activity. They found that the type of break in the DNA was critical: double stranded DNA with flush breaks was approximately three times more effective at activation than double strand breaks with projecting 3' termini, and about ten times more effective than single strand nicks or double strand breaks with projecting 5' termini.

To date, most studies carried out have focussed on damaging cellular DNA followed by investigation of the physical and biological effects resulting both in the presence and absence of ADPRT inhibitors.

Damage by alkylating agents (James and Lehmann, 1982; Skidmore *et al.*, 1979; Durkacz *et al.*, 1980), ionising radiation (James and Lehmann, 1982; Lunec *et al.*, 1983) and ultraviolet radiation (u.v.) (James and Lehmann, 1982; Cleaver *et al.*, 1983) have received most attention. DNA damage mediated activation of ADPRT has been shown experimentally to be accompanied by a concomitant fall in the

cellular NAD^+ level (Whish *et al.*, 1975; Smulson *et al.*, 1975; Wielckens *et al.*, 1982), supporting the earlier work of Roitt (1956), who had shown that alkylation damage was responsible for reduced NAD^+ levels leading to inhibition of glycolysis in ascites tumour cells. The depletion mechanism, however, was unclear. Activation of NAD^+ glycohydrolase, inhibition of NAD^+ biosynthesis, or possibly some other factor could have been responsible for the observed fall in concentration. Whish and co-workers (1975) showed that activation of ADPRT was, in fact, responsible, while it was subsequently demonstrated that specific inhibition of ADPRT could effectively block NAD^+ depletion.

Studies of the involvement of ADPRT on DNA repair have primarily concerned measurement of strand break differences thoughout defined experimental regimes. Sensitive methods such as nucleoid sedimentation (Cook and Brazell, 1975) and alkali elution (Cleaver *et al.*, 1985), and less sensitive methods such as sedimentation of DNA through alkaline sucrose gradients (Durkacz *et al.*, 1980) have been employed extensively, but all suffer from a similar shortcoming in that experimental observations reflect only a 'snapshot' of the balance between strand breakage and ligation processes (James and Lehmann, 1982). As a direct result, many experimental observations have conflicted.

Following alkylation damage, most workers have reported an increase in the level of strand breaks, in the presence of ADPRT inhibitors, relative to inhibitor free controls (Durkacz *et al.*, 1980, 1981; Cleaver *et al.*, 1983; James and Lehmann, 1982), although decreases have been observed in a few cases (Bohr and Klenow, 1981; Althaus *et al.*, 1982).

Net strand break rejoining has also been reduced in the presence of inhibitors following radiation with *X*-rays (Zwelling *et al.*, 1982; Mattern *et al.*, 1983) and Y-rays (Durkacz *et al.*, 1981), although this has not been shown to be a universal effect.

Following damage of HeLa cells by u.v. irradiation, Otsuka and co-workers (1986) observed no inhibitor mediated effects on repair metabolism, even in the presence of 10 mm 3AB, supporting a number of earlier observations (James and Lehmann, 1982; Cleaver *et al.*, 1983). However, the number of strand breaks induced by u.v. treatment is much lower than those induced by equitoxic doses of alkylation or ionising radiation (Shall, 1984). Collins (1987) therefore suggested that poly ADPR synthesis would only be seen in exponentially growing cells following u.v. treatment if DNA strand breaks were allowed to accumulate using suitable inhibitors. Otsuka and co-workers (1986), however, observed no 3AB mediated alteration in strand break numbers in the presence of hydroxyurea and 1-β-D-arabinofuranosyl cytosine and suggested that ADP-ribosylation was not involved in u.v. radiation induced DNA repair.

The involvement of ADP-ribosylation in a late stage of DNA excision repair, in particular ligation, was suggested largely as a result of demonstrations that ADPRT inhibition had little or no effect on incision, excision or repair synthesis processes (Durkacz *et al.*, 1980; Shall, 1984).

As stated previously, Creissen and Shall (1982) supported this hypothesis by demonstrating that in L1210 cells, following alkylation damage, DNA ligase II activity was elevated 2-3 fold and that this increase in activity was 3AB sensitive. However, DNA ligase II has

recently been shown to be inhibited *in vitro* following ADP-ribosylation (Yoshihara *et al.*, 1985). Furthermore, Cleaver's group showed that low concentrations of 3AB (<2 mM) in alkylation damaged human lymphoid cells led to decreased strand break frequencies (Cleaver *et al.*, 1985), while 3AB concentrations as high as 5 mM stimulated ligation of repair patches which had temporarily been held open by DNA polymerase α inhibitors (Cleaver and Park, 1986).

They proposed that the presence of 3AB was responsible for two related effects. First, the cellular ATP level, an essential co-factor for ligase II, was maintained, and second, the ADPR modulated inhibition of a number of DNA repair enzymes (Yoshihara *et al.*, 1985) was avoided. Indeed, this second factor could explain the increase in repair synthesis observed by some workers (Sims *et al.*, 1983; Cleaver and Morgan, 1985). *In vivo* inhibition of non specific Ca^{2+}/Mg^{2+} endonucleases by ADP-ribosylation could be relieved by modulation of ADPRT with 3AB. The resulting restoration of activity would allow random attack of DNA leading to an increase in total repair sites relative to the initial number of damage induced sites. A recent report by Adamietz (1987), however, showed that modified endonucleases were not detectable using boronate chromatography following DMS treatment of rat hepatoma cells.

Massive depletion of cellular NAD^+ levels, as a consequence of severe DNA damage, leading to cell death, has been postulated as a means of preventing neoplastic transformation in eukaryotes (Sims *et al.*, 1983; Berger, 1985). It may prove significant that prokaryotic organisms do not appear to possess poly ADP-ribosylation systems, as mutation in such organisms can only benefit the organism

in evolutionary or survival terms. In higher organisms, however, the long term effects of inefficient DNA repair processes can prove fatal. ADPRT mediated death of the damaged cell represents an effective preventive measure. The phenomenon has been termed the suicide response (Berger, 1985).

1.4.2 Cellular differentiation and proliferation

The alterations in gene expression associated with cellular differentiation involve a number of processes necessitating localised changes in chromatin structure. The possible introduction of DNA strand breaks by gene amplification and transposition mechanisms, for example, have led many workers to investigate possible alterations of ADP-ribose metabolism throughout cellular differentiation.

ADPRT inhibitors have again been an invaluable tool in many systems, although again a lot of the resulting data generated are conflicting.

Johnstone and Williams (1982) showed that in the presence of 3AB or 3MeB, mononuclear leukocyte differentiation, as measured by mitogen induced incorporation of [^{3}H]-thymidine, was blocked. Nucleoid sedimentation rates increased dramatically up to 8h following mitogen treatment and the observed decrease in net strand breaks this indicated was found to be susceptible to blocking by 3MeB. Further studies with a similar system by Ittel and co-workers (1983) partly reflected their observations, showing that the presence of 5 mM 3AB or 10 mM nicotinamide throughout mitogenic stimulation of human lymphocytes led to decreased DNA synthesis. However, cellular proliferation was also decreased, which conflicted with the observations of Johnstone and Williams, who, in non differentiating cells, failed

to see any inhibitor mediated alterations in cellular proliferation.

The differentiation of avian muscle cells, as determined by increased creatine phosphokinase activity and fusion of myoblasts into myotubes, was found by Farzaneh and co-workers (1982) to be sensitive to blocking by ADPRT inhibitors and nicotinamide deprivation. They also observed that the onset of differentiation was coincident with an increase in the frequency of single stranded DNA breaks. Hacham and Ben Ishai (1985) also found myoblast fusion to be nicotinamide and 3AB sensitive. They further observed that during rat myoblast fusion into myotubes a 3-fold transient stimulation of ADPRT activity occurred and that maximal sensitivity to the inhibitors, at a concentration of 10 mM, occurred immediately prior to fusion. At such inhibitor levels, no effect was observed on proliferation

The differentiation of limb mesenchyme cells has similarly been studied by a number of groups. Caplan (1985) was able to show a transient increase in ADPRT activity with initiation of differentation of limb mesenchyme cells into muscle and cartilage, whereas Nishio and co-workers (1983) observed that differentiation of chick embyro limb bud mesenchyme cells could be induced by BA, 3AB, 3MeB, or nicotinamide, to form chondrocytes. This study conflicted directly with the observations of Caplan, and a post commitment involvement of the inhibitors was postulated.

Williams (1985) and Al-Sharif (personal communication) have shown that transformation of *Trypanasoma brucei* from the non-mobile amastigote form to the flagellated trypomastigote can be reversibly blocked by ADPRT inhibitors and a novel means of controlling this parasite has been suggested. The validity of the use of

benzamide inhibitors in differentiation studies, however, has been questioned by some workers.

Brac and Ebisuzaki (1985) found that while DMSO induced differentiation of Friend cells could be inhibited by 3AB or 3MeB, high levels of inhibitor (20 mM BA) in the absence of DMSO could also induce differentiation. They suggested that only low levels of inhibitor were responsible for specific ADPRT modulation and that high levels are responsible for more widespread non specific effects, supporting the views of Cleaver (section 1.3.5).

As alterations in ADPRT activity throughout differentiation processes have been widely observed, it is surprising that accompanying changes in DNA strand break frequencies have not always followed. Indeed, Brac and Ebusuzaki (1985) found no alteration in strand break frequencies associated with ADP-ribosylation in Friend cell differentiation and suggested that any change in frequencies was either very small or very transient. Similarly, Althaus and co-workers (1982b) could not detect break frequency changes during neoplastic transformation of adult rat hepatocytes and Cleaver's group also suggested that DNA strand break changes were unlikely to be associated with malignant transformation of hamster embryo cells and mouse C3H 10T½ cells (Borek *et al.*, 1984).

The decreased potential of differentiating cells to repair DNA damage could explain increased break frequencies observed in some cell lines. This seems an unlikely explanation, however, as Farzaneh and co-workers have shown that differentiating chick myoblast cells (1982) recovering from Y-irradiation, and human HL60 cells (1987) repairing Y-irradiation and DMS induced damage, do not

suffer depressed DNA repair potential throughout differentiation.

An interesting observation made by Ikai and Ueda (1980), that ADPRT activity was present in monocyte and lymphocyte nuclei, but not granulocytes or erythrocytes, was intriguing in that both monocytes and granulocytes derive from the same precursor cells. Furthermore, Khan and Francis (1987) showed that induced differentiation of granulomonocytic precursors to monocytes could be blocked by ADPRT inhibitors. Two different mechanisms of strand break metabolism could be observed in the differentiating cell lines. Measured by nucleoid sedimentation, monocyte differentiation was associated primarily with ligation of pre-existing strand breaks, whereas granulocytic differentiation was accompanied by opening and closing of new strand breaks. Unlike the monocytic specific breaks, repair of those associated with granulocytic differentiation was insensitive to inhibitors of ADPRT. As well as being involved in differentiation at a general regulatory level, it was therefore proposed that ADP-ribosylation was involved in highly specific cell lineage switch mechanisms.

Finally, hormonal control of ADP-ribose metabolism has been proposed. Pekala and co-workers (1981) found that pre-adipocytes could be induced to differentiate to adipocytes by dexamethasone, insulin and methylisobutylxanthine and that differentiation was followed rapidly by a transient suppression of ADPRT activity, while in a more recent study, Bolander (1985), using α lactalbumin production as a marker of mammary gland differentiation, showed that ADPRT inhibition stimulated differentiation. Prolactin suppressed ADPRT activity and insulin was found to stimulate ADPR glycohydrolase

activity. Insulin mediated fluctuations in cAMP levels were postulated as a means of modulating polymer turnover *via* glycohydrolase. Furthermore, changes in ADPR metabolism were subsequently found to be unlikely to be associated with DNA synthesis, but likely to be linked to differentiation (Bolander, 1986).

1.4.3 The cell cycle

Considering the vast reorganisation of chromatin involved, it is hardly surprising that ADP-ribosylation has been shown to vary significantly throughout the cell cycle of eukaryotes. Kidwell and Mage (1976) observed an increase in ADPR polymer formation throughout S phase to a peak at the S/G2 bounday in HeLa cells.

Adolph and co-workers (Adolph and Song, 1985; Adolph, 1985; 1987a,b; and Song and Adolph, 1983) have extensively studied the specificity and degree of modification of a number of receptor species in the HeLa cell cycle and essentially support the above finding, observing an overall 2-fold increase in modification during S phase. The levels of modification of the structural nuclear lamins, and ADPRT itself, however, remain fairly constant throughout (Adolph and Song, 1985). One and two dimensional electrophoretic analysis of labelled receptors using either [^{3}H] adenosine or [^{32}P] NAD^{+} as a precursor, showed essentially similar modification patterns (Song and Adolph, 1983; Adolph. 1987a,b). Histones were extensively modified to similar levels at all stages of the cycle, the only marked differences concerning non histone species. Striking differences were observed when the modification of metaphase chromosomal proteins, representing the stage when the chromatin is at its most condensed, was contrasted with that of interphase nuclei, when

the chromatin is at its most relaxed. Whereas only the polymerase, of the non histone proteins, was modified significantly in metaphase chromosomes, over 100 species of non histone receptor species could be identified in interphase nuclei (Adolph, 1985, 1987b). Cell cycle changes in modification were not sensitive to alteration by inhibition of DNA, RNA or protein synthesis, suggesting that cell cycle ADP-ribosylation is not under genetic control. Stimulation of metaphase ADP-ribosylation by either nuclease treatment, to induce strand breaks, or depletion of Mg^{2+} ions, to relax the chromosomes allowing increased accessibility, did not lead to significant changes in modification resembling that of interphase nuclei (Adolph, 1987a).

In contrast, however, Tanuma and co-workers (1978) found peak ADPRT activity at mitosis, although they did report high activity at the S/G2 border in synchronised HeLa cells. They were also able to show that increased modification was concomitant with chromosome condensation and decreased transcription, observations supported by direct and indirect immunofluorescence studies utilising antibodies raised against poly ADPR (Kanai *et al.*, 1981).

More recently Tanuma *et al.*, (1986b), studying receptor species, found that modification of HMG proteins 14 and 17 and histone H1 was increased 12- and 5-fold respectively in mitosis, compared to G1 nuclei. The increased modification at metaphase also reflected average chain lengths of 10.4 and 17.2 for histone and non histone proteins respectively, compared to 3.0 and 6.1 in G1 nuclei. This represented an overall 3-fold increase in chain length in metaphase chromosomes.

The glucocorticoid regulated synthesis of specific mRNA species, coupled with the observation that degradation of ADPR polymer attached

to HMG 14 and 17 can be glucocorticoid mediated prompted the suggestion that modification of HMG 14 and 17 could be associated with switching off transcription (Tanuma and Johnson, 1985). In conflict with the *in vitro* observations of Poirier's group (see section 1.3.6), they also suggested that increased histone H1 modification was associated with chromatin condensation during mitosis.

Considering that the two groups (Adolph and Tanuma) used the same cell line to study the same process, the conflict of results is somewhat surprising. Whether the differences are attributable to the use of thymidine, a recognised inhibitor of ADPRT, to synchronise the cells, by Adolph, or to some other factor, is difficult to ascertain. The differences do, however, highlight once more the difficulties encountered in obtaining comparable results for ADPR metabolism.

1.5 Rationale for the Project

As discussed in section 1.3.5, there are a number of potentially serious drawbacks associated with the use of inhibitors as probes for ADP-ribosylation systems.

Recently, technology developed by Izant and Weintraub (1984) has made possible mono specific inhibition of gene products at a pre-, rather than post-, translational level.

The principle involved is simple and extremely elegant. In normal genetically-active cells, a dynamic pool of coding or 'sense' mRNA species is present resulting from transcription of the coding strands of the DNA duplexes. The new methodology introduces 'antisense' mRNA species into the pool, which is then allowed to

anneal to the sense mRNA by virtue of its complementarity. Processing and ultimately translation of the message, may be hindered as a result, depending on the size, the position on the sense transcript at which the complementary antisense species binds, and the ratio of sense to antisense species.

The antisense species are synthesised by virtue of transfection of the cell line with suitable expression vectors into which the sequence of interest has been spliced. Further refinement is offered by the availability of inducible vectors. In the case of ADPRT, switching translation on and off provides an excellent means of studying the effects of enzyme deficiency directly [for review, see Izant and Weintraub (1985)]. The aim of the work described in this thesis was to test the applicability of antisense mRNA technology to study of the ADP-ribosylation system.

Central to the project was the purification to homogeneity of ADPRT in quantities large enough to raise polyclonal serum. Although the enzyme has been extensively purified by many groups of workers (see Table 1), none of the published protocols has been widely adopted, suggesting either unreliability and/or laborious time-consuming methodology.

Antiserum may be used to probe cDNA libraries, but the immunoprecipitation of polysomes, followed by purification of mRNA, was proposed as the time-consuming construction and screening associated with such approaches is not required. cDNA synthesised from the isolated message could then be used directly in antisense studies.

With knowledge of the genetic code and codon usage of the organism from which the enzyme was isolated, polypeptide sequence data can also be used to synthesise corresponding oligonucleotide sequences. The incorporation of such sequences into expression vectors and their subsequent use as anti ADPRT mRNA probes was considered.

CHAPTER 2

Routine Methodology

Presentation of Results

Although the immunochemical studies carried out were suitable for presentation in the established format of methodology followed by results, the developmental nature of the enzyme purification was such that it was deemed more expedient to present that section of the work in the form of a chronological account.

The above two sections are preceded by the well established analytical techniques which were routinely used throughout.

2.1 Nuclear ADPRT Assay

ADPRT activity was measured as the incorporation of [2-^{3}H-ade]-NAD^+ into trichloroacetic acid (TCA) insoluble material.

The assay mixture contained 100 mM TEA.HCl pH 8.0, 10 mM $MgCl_2$, 2 mM DTT, 500 nM NAD^+ (2 µCi/nmole) in an incubation volume of 480 µl. When necessary, the assay was supplemented with 40 µg sonicated calf thymus DNA and 40 µg calf thymus histones. The assay mixture was pre-incubated at 26 °C and the reaction started by addition of 20 µl enzyme solution. Incubation was carried out at 26 °C for the required time period (usually 5 min), after which the reaction was stopped by one of the two methods described below:

Method 1

20 µl aliquots of the reaction mixture were spotted onto dried Whatman 3MM paper discs, pre-washed in 20% w/v TCA in diethyl ether. After drying at room temperature, the discs were washed in ice cold 5% w/v TCA for 4 x 15 min, followed by a 5 min wash in 95% v/v ethanol. After drying, the discs were transferred to scintillation vials, face

up, and counted for radioactivity immersed in 3 ml 'Optiphase' scintillation cocktail (LKB).

Method 2

125 µl ice cold 100% w/v TCA in water was added to the mixture, followed by vortexing. The tubes were then placed on ice for a minimum of 30 min, after which the entire contents were filtered under vacuum through a Whatman GF/C disc. The tubes were rinsed twice with about 1.5 ml cold 5% w/v TCA and the discs washed by passing 4 x 5 ml cold 5% w/v TCA through the disc. After a final 5 ml 95% v/v ethanol wash, the discs were dried and counted for radioactivity as method 1.

The NAD^+ concentration used was approximately 2 orders of magnitude below the K_m of the enzyme. A higher concentration was used for the yield calculations (see later).

Method 1 is useful where a time course study is required. The method facilitates quantitation of polymer at intervals of only a few seconds and is ideal for kinetic studies. Processing of large numbers of samples is also possible in a relatively short time period and accordingly column chromatography fractions were assayed by this method. Method 1 suffers, unfortunately, in that aliquots taken from the same reaction mixture are not independant evaluations and are therefore statistically limiting. Method 2, however, allows meaningful statistical evaluations to be carried out, and collects all products within limitations of size. In order to make statistically valid statements, however, the measurement has to be carried at least three or four times and is therefore time consuming. Method 2 was used for the yield calculations described later and, when

numbers were not restrictive, for column fraction evaluations. Throughout the developmental stages, unless stated otherwise, enzyme activity values refer to the total TCA precipitable material in each fraction under standard 5 min assay conditions. Only with quantitative determinations, such as yield assessment, are activities quoted as nmoles of product per min per mg protein.

2.2 Protein Estimation

Unless otherwise stated, the method of Bradford (1976) was used. The assay has the advantages of being rapid to carry out, sensitive, reproducible and relatively free from interference by contaminating reagents.

The protein reagent was prepared by dissolving 100 mg Coomassie Brilliant Blue G-250 (Sigma) in 50 ml 95% v/v ethanol. 100 ml 85% w/v orthophosphoric acid was then added and the mixture diluted to a final volume of 1 litre. The solution was filtered through Whatman No.1 paper, under gravity, before use.

50 µl of protein sample were made up to 100 µl with 0.1 M Tris.-HCl pH 7.5 to which was added 5 ml of protein reagent. The solution was mixed by gentle inversion or vortexing, taking care to avoid foaming, and after standing for 5-60 min at room temperature, the optical density was read at 595 nm. Values were typically obtained from a 0-100 µg bovine serum albumin standard curve.

2.3 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

When placed in an electric field, proteins will exhibit differential mobilities dependant on size, shape and charge. When exploited in conjunction with the sieving effect of a porous inert

matrix, such as agarose or polyacrylamide gel, unparalleled separation of protein species is possible.

The use of the anionic detergent sodium dodecyl sulphate (SDS) to pretreat proteins prior to electrophoresis is routinely used to effect separation primarily on the basis of uniform size to charge ratios. Most proteins react with SDS to form regular, solubilised rod-like protein-detergent complexes. The amount of SDS interacting with proteins is usually proportionate to the size of the molecule and effectively removes native charge and shape contributions from the separation.

Electrophoresis coupled to SDS treatment is undoubtedly one of the most powerful analytical techniques available in protein biochemistry. As well as the high resolution attainable, the technique is relatively quick and very sensitive (when used in conjunction with silver staining or autoradiography), while allowing analysis of many samples simultaneously.

The system developed by Laemmli (1970) was used throughout, using either 7.5%, 10.0%, or 5-20% w/v polyacrylamide gradient resolving gels.

The resolving gel was made by mixing aliquots of stock acrylamide (T = 30%, C = 2.67%), dependant on the porosity of the gel required, with 3.75 ml stock resolving buffer (3 M Tris.HCl pH 8.8), 0.3 ml 10% w/v SDS, 1.5 ml ammonium persulphate solution (1.5% w/v, made freshly each time) and distilled H_2O to 30 ml. 15 µl *N,N,N',N'*-tetramethylethylenediamine (TEMED) was mixed with the gel solution and the gel cast. A small aliquot of butan-2-ol was layered onto the top of the gel and polymerisation allowed to take place at room temperature (approximately 30-60 min). After setting the butan-2-ol

or gel buffer ?!

was removed and the top of the gel rinsed with distilled H_2O prior to pouring the stacking gel.

The stacking gel was made by mixing 2.5 ml stock acrylamide, 5 ml stock buffer (0.5 M Tris.HCl, pH 6.8), 0.2 ml 10% w/v SDS, 1.0 ml ammonium persulphate solution and 11.3 ml distilled H_2O. 15 µl TEMED was added, followed by brief mixing. The gel was poured onto the top of the resolving gel and a comb with the required number of wells inserted. Both reservoir buffers were 25 mM Tris, 192 mM glycine, 0.1% w/v SDS.

Samples were prepared by addition of an equal volume of 2 x gel sample buffer (0.25 M Tris.HCl, pH 6.8; 4% w/v SDS; 10% w/v β-ME; 20% v/v glycerol; 0.002% w/v bromophenol blue) and incubation in a 100 °C water bath for 3 min. The tubes were cooled to room temperature prior to centrifugation for 3 min at high speed in a microfuge. After loading, the samples were electrophoresed at a constant 100 V until the bromophenol blue dye front reached the anodal end of the gel slab.

2.4 Staining of Gels Post Electrophoresis

2.4.1 Coomassie Brilliant Blue R-250 (Sigma)

The gel was immersed in stain solution (0.1% w/v Coomassie Brilliant Blue R-250, 50% v/v methanol, 10% v/v acetic acid) and agitated gently for a minimum of 2 h at room temperature. Non protein bound stain was removed by immersion in successive changes of destaining solution (5% v/v methanol, 7% v/v acetic acid) at room temperature until a clear background was observed.

2.4.2 Silver staining

Standard methods of protein staining like Coomassie Blue are satisfactory in the majority of situations. However, when the availability of protein is limiting and sub-microgram quantities are being analysed, such techniques are of limited use due to their relative insensitivities (0.1 to 1.0 μg). The development of silver staining techniques, with sensitivies estimated between 20-200 times greater than Coomassie Blue, has partly alleviated such problems. Silver staining of gels was used as minimal losses of purified fractions were essential.

The method of Merril and co-workers (1981) was used, which is quoted as detecting 0.1 μg BSA.

All manipulations were carried out wearing disposable gloves and the gels were agitated end to end at room temperature throughout.

Immediately post electrophoreses, the gel was placed in 12% v/v acetic acid and incubated overnight. The fixed gel was then washed in 10% v/v methanol, 5% v/v acetic acid for 3 x 10 min, followed by incubation in a solution of 3.4 mM $K_2Cl_2O_7$, 3.2 mM HNO_3 for 1 h. After four quick distilled H_2O rinses (approximately 30 s each), the gel was incubated in a freshly made solution of 12 mM $AgNO_3$ (Analar-BDH) for 1 h in the dark. The $AgNO_3$ solution was then poured away and the gel quickly rinsed twice with developer (30 g Na_2CO_3, 1.5 ml formaldehyde solution per litre, warmed to 40 °C). A third volume of developer was added and agitation continued until the required stain intensity was reached. The developer was discarded and the reaction stopped by addition of 5% v/v acetic acid. Stained gels were stored in distilled H_2O.

In some cases, a quicker method of fixing was employed. Instead of incubation overnight in 12% v/v acetic acid, the gel was incubated for 20 min at room temperature in 50% v/v methanol/12% v/v acetic acid followed by 3 washes in 10% v/v ethanol/12% v/v acetic acid. All further steps were unchanged.

2.5 Thin Layer Chromatography (TLC)

The support used throughout all TLC studies was nylon backed polyethyleneimine (PEI) cellulose which acts as a strongly basic anion exchanger.

Three solvent systems were employed in developing the chromatograms:

1. Butanol:H_2O:methanol:NH_3 (60:20:20:1)

   The highly organic nature of the solvent reduces hydrophobic interactions. Separation is therefore mainly on the basis of size and charge.

2. 200 mM $LiCl_2$

   Increasing ionic strength decreases electrostatic interactions and in some cases hydrophobic interactions. In 200 mM $LiCl_2$, therefore, separation is mainly on the basis of size.

3. 100 mM Acetic acid

   In 100 mM acetic acid, the PEI will be protonated. Charge interactions will therefore be the predominant factor.

CHAPTER 3

Non-affinity Enzyme Purification

3.1 Preparation and Salt Extraction of Pig Thymus Nuclei

Pig thymus glands were collected immediately post slaughter from the abattoir and immersed in ice. Fat and connective tissue were removed and conveniently sized portions stored at -20 °C until required.

For 12 g tissue, the following protocol had been routinely used in this laboratory and was subsequently used for all preliminary studies.

While still frozen, the tissue was shaved into thin slices with a scalpel and placed in 200 ml ice cold homogenising buffer (12 mM Tris.HCl pH 7.5, 0.32 M sucrose, 3 mM $MgCl_2$, 5 mM β-ME). Homogenisation was carried out for 3 x 30 s in a Sorvall Omnimixer (Dow Chemicals) at setting 2.5. After filtration through one layer of muslin, the homogenate was centrifuged for 15 min at 4 °C at 5000 rpm in an MSE 18 centrifuge. The pelleted nuclei were resuspended in 10 ml 20 mM Tris.HCl pH 7.5, 0.15 M NaCl, 5 mM β-ME. Equal aliquots of nuclei suspension were then transferred to 5 ml cellulose nitrate ultracentrifuge tubes, followed by addition of an equal volume of 0.1 M TEA.HCl pH 8.0, 1 M NaCl, 10 mM $MgCl_2$, 5 mM β-ME. The tubes were mixed gently by inversion, balanced, and centrifuged at 4 °C for 2 h at 225000 g. The supernatant was decanted from the gelatinous pellet, typically 50% of the tube volume, and the extract frozen in aliquots at -20 °C until required.

Presence of DNA in the salt extract

One of the most difficult aspects of purifying a chromatin bound protein is separation of the protein from the DNA with which it is intrinsically associated.

Salt extraction of nuclei followed by high speed centrifugation removes most of the DNA, but leaves residual levels too high for scaled up column chromatography. If this DNA is not removed, re-association of DNA and protein may occur, particularly if the salt concentration is reduced by dilution, or a desalting step. Such reassociation into heterogenous DNA/protein complexes can cause variable behaviour when separation is attempted, at least partly, on the basis of charge.

Accepted methods of DNA removal such as protamine sulphate (Moss *et al.*, 1976) and streptomycin sulphate precipitation are not applicable in this case, due to their dependance on low ionic strength.

The strong negative charge held by DNA at physiological pH suggested that anion exchange chromatography could be a successful means of separating DNA from ADPRT.

3.2 Ion Exchange Chromatography

Ion exchange chromatography separates molecules on the basis of differences in charge density and is divided into two categories:

(a) Anion exchange chromatography

The chromatographic support carries a net positive charge at the chosen pH. Molecules with a P_i above this pH carry a net negative charge and may interact with the support. Gradual disruption of such interactions, by increasing the concentration of salt, for example, passing through the support, causes

sequential detachment of bound molecules. Those with the lowest net charge emerge first, followed by those of increasing charge density.

(b) Cation exchange chromatography

This is exactly the converse to anion exchange. The support holds a net negative charge and positively charged ions interact.

3.2.1 Labelling of L1210 cells with [5-^{3}H]-thymidine

Although the amount of nucleic acid present in the salt extract was sufficient to impair column flow rates, the amounts were too low to be detected by standard DNA assays (Setaro and Morley, 1977; Richards, 1974). To overcome this, L1210 cells were grown in the presence of tritiated thymidine to label the DNA and a crude extract of the labelled cells made. Assuming that murine DNA behaved similarly to the pig thymus DNA, it was therefore possible to monitor the behaviour of the total DNA throughout further processing of the pig thymus nuclear extract.

To 30 ml RPMI containing 2 x 10^5 L1210 cells per ml was added 100 µl [5-^{3}H]-thymidine (Amersham - 47.5 Ci/mmol, 1 mCi/ml). The cells were incubated for 18 h at 37 °C until they were in mid log phase, equivalent to one doubling time. The cells were spun down at 1000 rpm for 5 min in a bench centrifuge, followed by two washes with serum free RPMI. The cells were then suspended in 750 µl 8 M urea, 2 M NaCl, followed by a high speed microfuge spin for 5 min. 2 x 5 µl were counted for radioactivity after precipitation on acid washed discs and 0.94 µCi was found to have been incorporated.

3.2.2 Anion exchange chromatography of salt extracted nuclei

When dissociated from DNA, nuclear ADPRT would not be expected to bind anion exchange supports as the enzyme carries a net positive charge at physiological pH (see P_i later). Extraction of ADPRT by the method described is analogous to ion exchange chromatography: the DNA acts as a cation exchanger from which protein is eluted with increased salt concentration.

An anion exchange resin was therefore sought which was capable of binding DNA at a salt concentration capable of maintaining enzyme/DNA dissociation.

3.2.2.1 Aminoethyl cellulose chromatography - Aminoethyl cellulose is an anion exchange resin with a pK of between pH 9-10. The resin was prepared according to the manufacturers (Sigma) instructions and equilibrated in 0.1 M TEA.HCl pH 8.0, 10 mM $MgCl_2$, 5 mM β-ME. 0.027 μCi L1210 extract was added to 150 μl nuclear extract and diluted to 2 ml with equilibration buffer. The mixture was applied to a 1 ml aminoethyl cellulose column (5.0 x 0.5 cm) and allowed to flow through under gravity. The effluent was collected and the column washed with 3.2 ml equilibration buffer. 2 ml aliquots of eluting buffers (equilibration buffer supplemented with increasing concentrations of NaCl) were then passed through the column. The linear flow rate was approximately 35 cm/h throughout. All fractions were counted (0.3 ml x 5) for radioactivity in 3 ml Optiphase (Figure 2).

All of the DNA applied to the column was retained and 11% was eluted in the wash step. Between 0.3 and 1.0 M salt, all of the remaining DNA was recovered with peak elution occurring with the 0.5 M salt wash (57%).

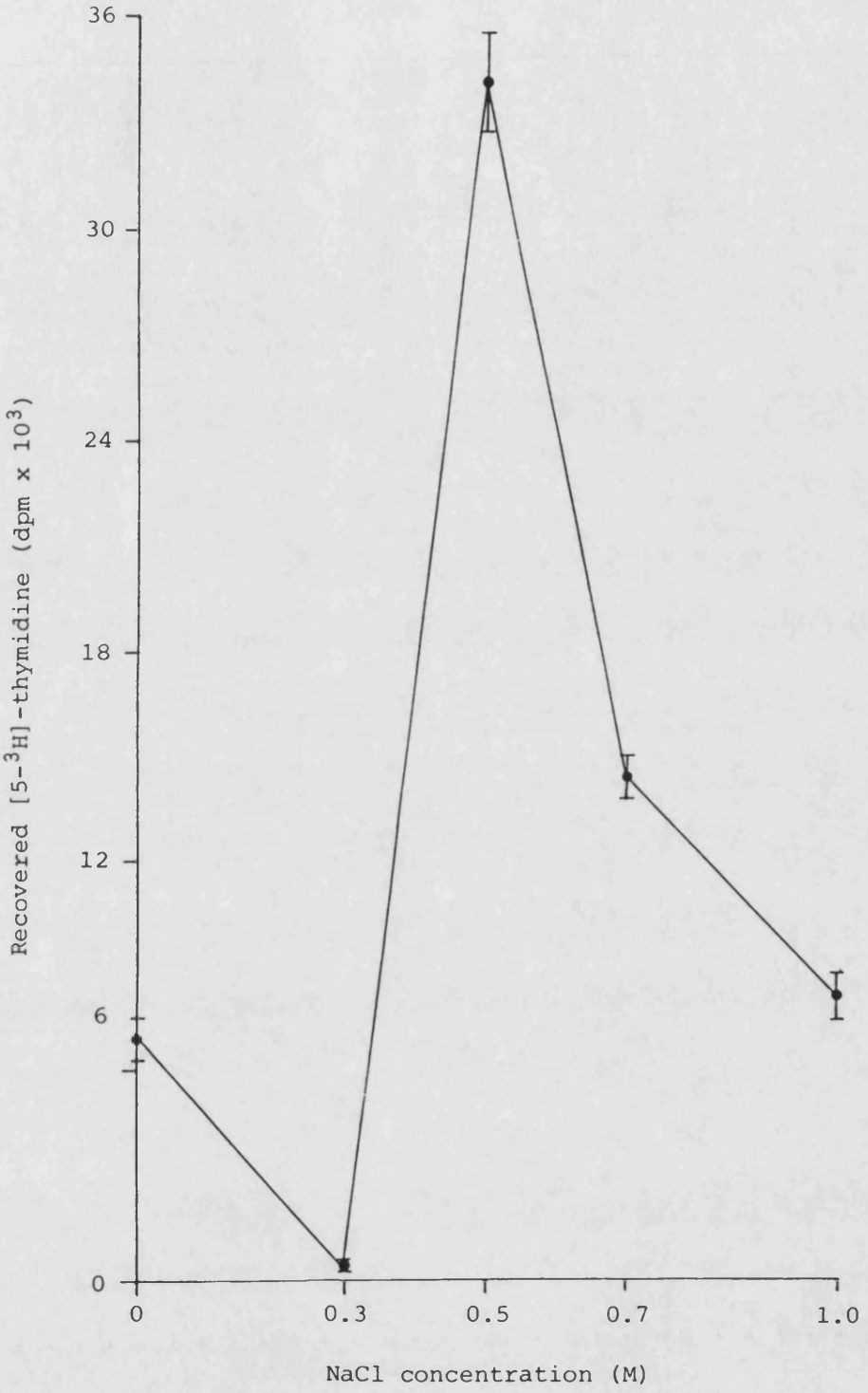


Figure 2 Aminoethyl cellulose chromatography of combined murine L1210 and pig thymus nuclear extracts.

0.027 µCi L1210 extract (section 3.2.1) was mixed with 150 µl thymus extract and chromatographed on a 1 ml aminoethyl cellulose column (see text for details).

As elution of DNA was taking place above 0.3 M salt, the resin was considered unsuitable for application in this case.

3.2.2.2 BioGel TE-2 chromatography - BioGel TE-2 (BioRad) an acrylamide based anion exchanger, was similarly tested for its DNA removing capacity. The adsorbant was prepared and equilibrated as previously, according to the manufacturer's instruction.

0.042 µCi L1210 extract was mixed with 100 µl nuclear extract and diluted to 2 ml with equilibration buffer. The mixture was then applied to a 1 ml BioGel TE-2 column and eluted as previously (Figure 3).

Again, all of the DNA was apparently retained by the column, although 12% was recovered in the wash step. No DNA was observed in the 0.3 and 0.5 M washes, however, which was very encouraging. 60.4% of bound DNA was recovered in the 0.7 and 1.0 M salt washes.

Column BioGel TE-2 chromatography of salt extracted nuclei

The previous experiment had shown that DNA remained bound to the column under conditions similar to those employed for nuclei extraction. The following experiment was carried out to ascertain the behaviour of the enzyme in the system.

A 1 ml BioGel column was set up and equilibrated with buffer as previously (0.1 M TEA.HCl pH 8.0; 10 mM $MgCl_2$; 5 mM β-ME). 1 ml of nuclear extract, diluted to 10% of the original salt concentration, was applied repeatedly to the column for 30 min, after which the column was washed with 2 ml equilibration buffer. Elution was attempted by addition of 1 ml aliquots of equilibration buffer, containing increasing salt concentrations, to the top of the column. Each fraction was assayed in triplicate for enzyme activity.

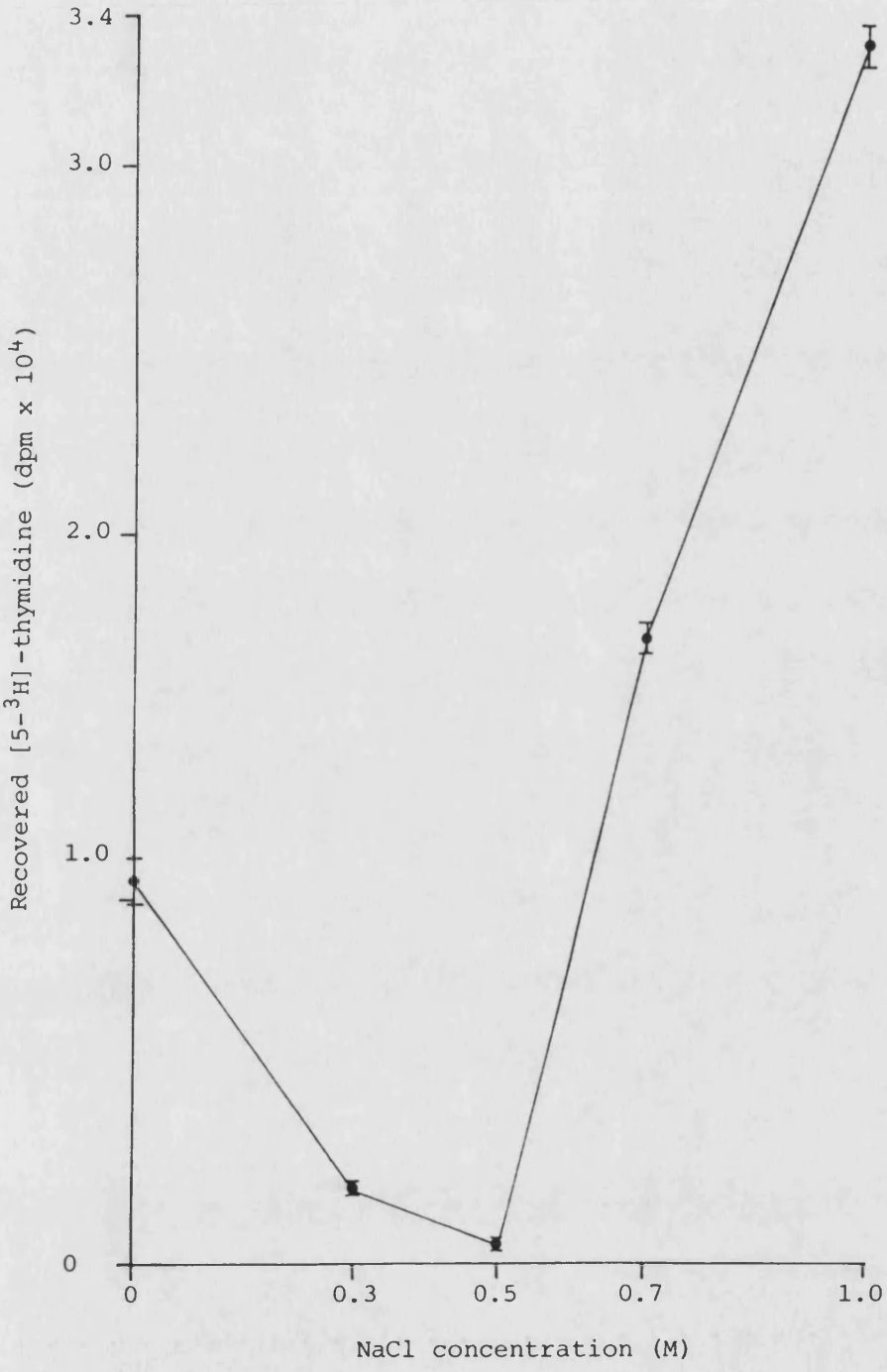


Figure 3 BioGel TE-2 chromatography of combined murine L1210 and pig thymus nuclear extracts.

0.042 µCi L1210 extract was mixed with 100 µl thymus extract and chromatographed on a 1 ml BioGel TE-2 column (see text for details).

It was very surprising to note that of 1.25×10^6 dpm applied to the column, 78% (0.98×10^6 dpm) of the enzyme activity was not retained by the column. As the experiment was carried out under identical conditions to the previous separation, it appeared that dilution to 50 mM salt was not immediately followed by DNA-protein reassociation. The remaining enzyme activity was not recovered in the salt elution.

Batch BioGel TE-2 chromatography

In the small scale developmental studies described thus far, column clogging and the time periods required for loading of material had not proved restrictive. However, in scaling up the preparation, this would not hold true. As BioGel TE-2 chromatography of DNA and ADPRT had proved successful in only one simple step, the possibility of batch separation was examined.

Two tubes were set up, each containing 4 ml diluted nuclear extract. To tube A was added 2 ml packed volume BioGel TE-2 in 8 ml total equilibration buffer and to tube B 8 ml equilibration buffer. The tubes were mixed gently at 4 °C for 15 min, after which they were spun at 4000 rpm on a bench centrifuge for 5 min. The supernatants were then assayed for enzyme activity and protein content.

Tube	dpm $\times 10^6$ recovered	µg protein	dpm $\times 10^3$/µg
A	2.84 ± 0.22	952	2.98
B	2.26 ± 0.25	1392	1.62

The batch procedure described resulted in an approximate 1.8 fold increase in specific activity. BioGel TE-2 treatment also resulted in a 25% elevation in total enzyme activity, probably due to binding of glycohydrolase to the adsorbant.

An identical experiment carried out with labelled L1210 extract plus nuclear extract successfully removed 75% of the radiolabelled material. Although this was not ideal, it was considered useful, particularly because BioGel TE-2 treatment appeared to remove glycohydrolase and/or phosphodiesterase as well as the majority of the DNA.

3.3 Hydroxyapatite (HA) Chromatography

3.3.1 Introduction

HA was first developed as a protein separation medium in Tiselius' laboratory (Tiselius *et al.*, 1956). Initially workers were reluctant to use the medium, partly because the chemistry involved was not well understood, and partly because behaviour varied vastly from preparation to preparation. Better understood, well established techniques such as ion exchange and gel filtration chromatography were therefore preferred.

The introduction of commercially produced HA, which behaved more consistently, led to the increasingly widespread use of the medium, particularly for proteins, and more recently as a separation technique applicable to nucleic acids. Its versatility and good resolving capabilities have led to the recognition of HA chromatography as a technique which augments, rather than competes with, traditional separation media.

Preparation of HA

HA is prepared commercially by the method of Tiselius by boiling brushite ($CaHPO_4.2H_2O$) with dilute NaOH. The resulting precipitate of HA [$Ca_{10}(PO_4)_6(OH)_2$] takes the form of long blade like crystals. The material is amphoteric with HA from different preparations possessing P_i values from pH 6.5 to pH 10.2.

HA-protein interactions

Bernardi (1971) was unable to offer a hypothesis, except to suggest that Ca^{2+} ions on the crystal surface are probably the site of binding of negatively charged carboxyl groups on the proteins. Tiselius had first noticed involvement of Ca^{2+} by observing reduced interaction with proteins in the presence of agents with high calcium affinity like citrate. No binding sites for interaction of positively charged protein groups were proposed.

More recently, Gorbunoff (1984) carried out an extensive study on the behaviour of a number of proteins with respect to P_i (pH 3.5 to pH 11), nature of eluant, protein structure and role of acidic and basic groups and proposed a hypothesis for HA/protein interactions. Interaction could not be explained solely by P_i. Basic proteins interact primarily by electrostatic attraction of the amine groups to the general negative charge of the column phosphates. Elution may be achieved by specific displacement with Ca^{2+} or Mg^{2+} ions or with F^-, Cl^-, ClO_4^-, SCN^- and PO_4^- ions. Acidic proteins interact specifically by complexing of free carboxyls with Ca^{2+} ions on the column. Binding and elution is therefore a consequence of complex interaction between specific and general electrostatic effects.

3.3.2 Binding of ADPRT to HA (BioGel HTP-BioRad)

5 ml diluted nuclear extract was added to 1 ml, packed volume BioGel TE-2 slurry and mixed gently on ice for 15 min. The slurry was pelleted in a bench centrifuge and the supernatant transferred to 1 ml of packed HA slurry in the same buffer. Mixing and centrifugation were repeated as for the BioGel TE-2 step.

5 ml extract was also treated with HA, omitting the BioGel TE-2 step.

The crude extract and all supernatants were assayed for enzyme activity and protein content.

Sample	Enzyme Act (dpm x 10^5)	Protein (μg)	Specific Activity (dpm/μg)
Nuclear extract	5.00 ± 0.80	1215	411
Post BioGel TE-2	7.66 ± 0.42	540	1418
Post HA	0	90	-
Post HA only	0	180	-

In this case the BioGel TE-2 step results in an apparent 56% gain in enzyme activity and a 3.5 fold purification. With the removal of the majority of residual DNA in this step, the extract appeared suitable for further processing *via* column chromatography.

It was also noted that all enzyme activity bound HA, regardless of whether the extract had been pretreated with BioGel TE-2.

3.3.3 Elution of ADPRT from HA

For basic proteins, the best methods of elution are competition with moderate phosphate concentrations or low levels of Ca^{2+} ions. In this case, stepwise elution using increasing phosphate levels was attempted.

Ten tubes were set up, each containing 0.5 ml packed HA equilibrated with TEA equilibration buffer. 2.5 ml nuclear extract were added to each tube followed by gentle mixing for 15 min. The HA was then pelleted in a bench centrifuge and the supernatants assayed for enzyme activity and protein content.

3 ml aliquots of potassium phosphate buffer, pH 8.0, containing 5 mM β-ME, of increasing concentrations were then added to each of the ten tubes, followed by gentle mixing for 15 min. The HA was pelleted again and all supernatants assayed for enzyme and protein (see Figure 4).

Of 4.07 x 10^5 dpm applied to the HA, 96% of the activity bound, of which 82% was recovered with a phosphate concentration of 0.5 M, 71% of the applied protein was retained. The specific activity of the 0.5 M fraction was 1.18 x 10^3 dpm/μg compared to the nuclear extract with 0.97 x 10^3 dpm/μg.

It was therefore possible to recover HA bound ADPRT with good yield.

3.3.4 Small scale HA chromatography - behaviour of residual DNA

In order to ascertain the behaviour of the residual DNA in the extract after treatment with BioGel TE-2, and the possible potential of HA as a purification step, radiolabelled L1210 extract supplemented with salt extract of nuclei was chromatographed on a small HA column.

2 ml nuclear extract plus 0.4 μCi L1210 extract were mixed and diluted to 20 ml with equilibration buffer. The diluted extract was loaded onto a 2 ml HA column followed by a 10 ml equilibration buffer wash. Elution was then attempted with stepwise increases of 50 mM potassium phosphate, pH 8.0, from 50 mM to 500 mM. 2 ml of each buffer solution were used, supplemented with 5 mM β-ME, and all fractions assayed for radioactivity, enzyme activity and protein content (Figure 5).

Of 2.2 x 10^6 dpm bound to the column, 2.6 x 10^6 dpm were recovered, again due probably to removal of polymer degrading enzymes. Peak enzyme activity was recovered at 0.4 M phosphate with the fraction

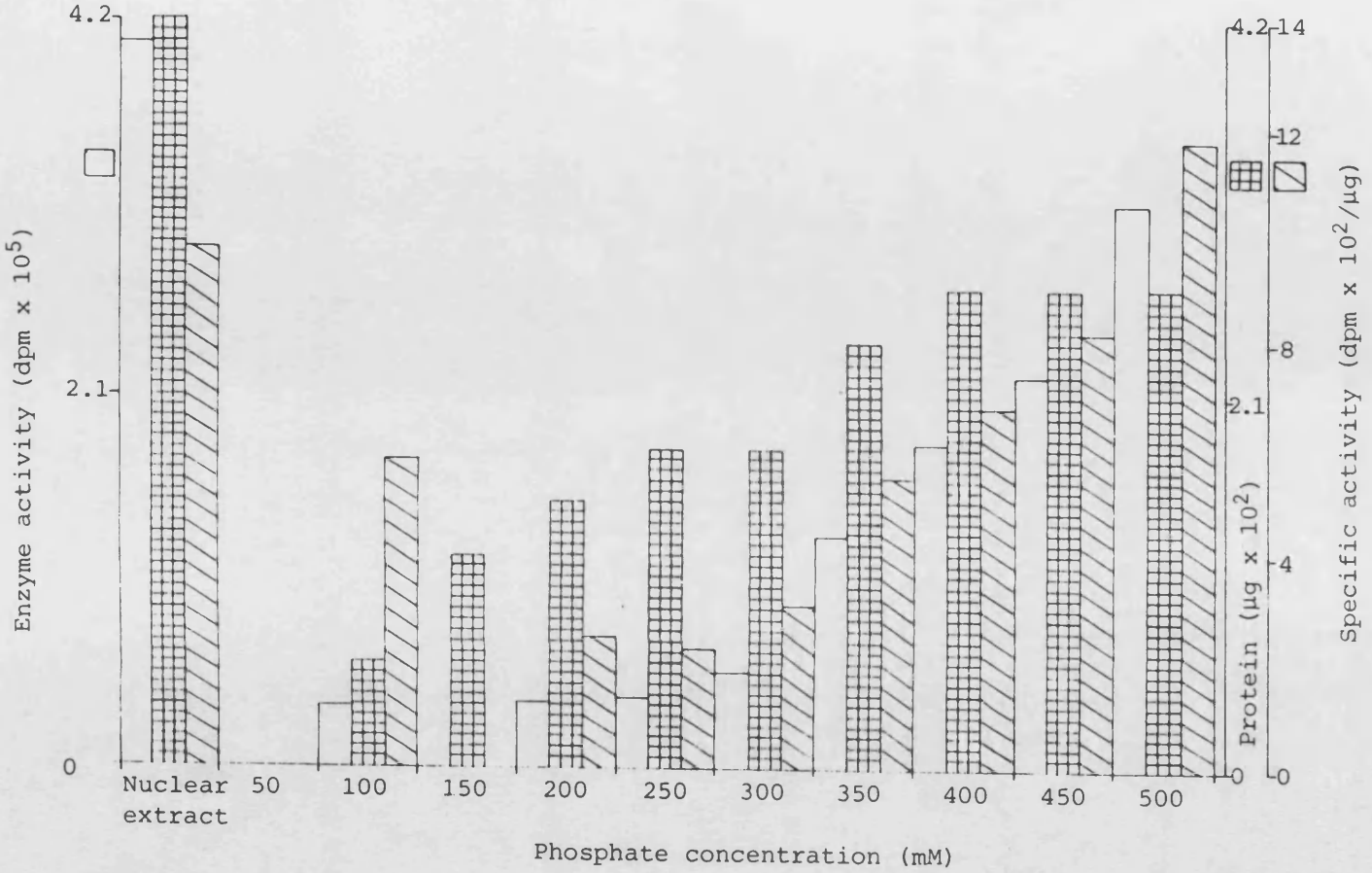


Figure 4 HA batch chromatography of nuclear extract (section 3.3.3).

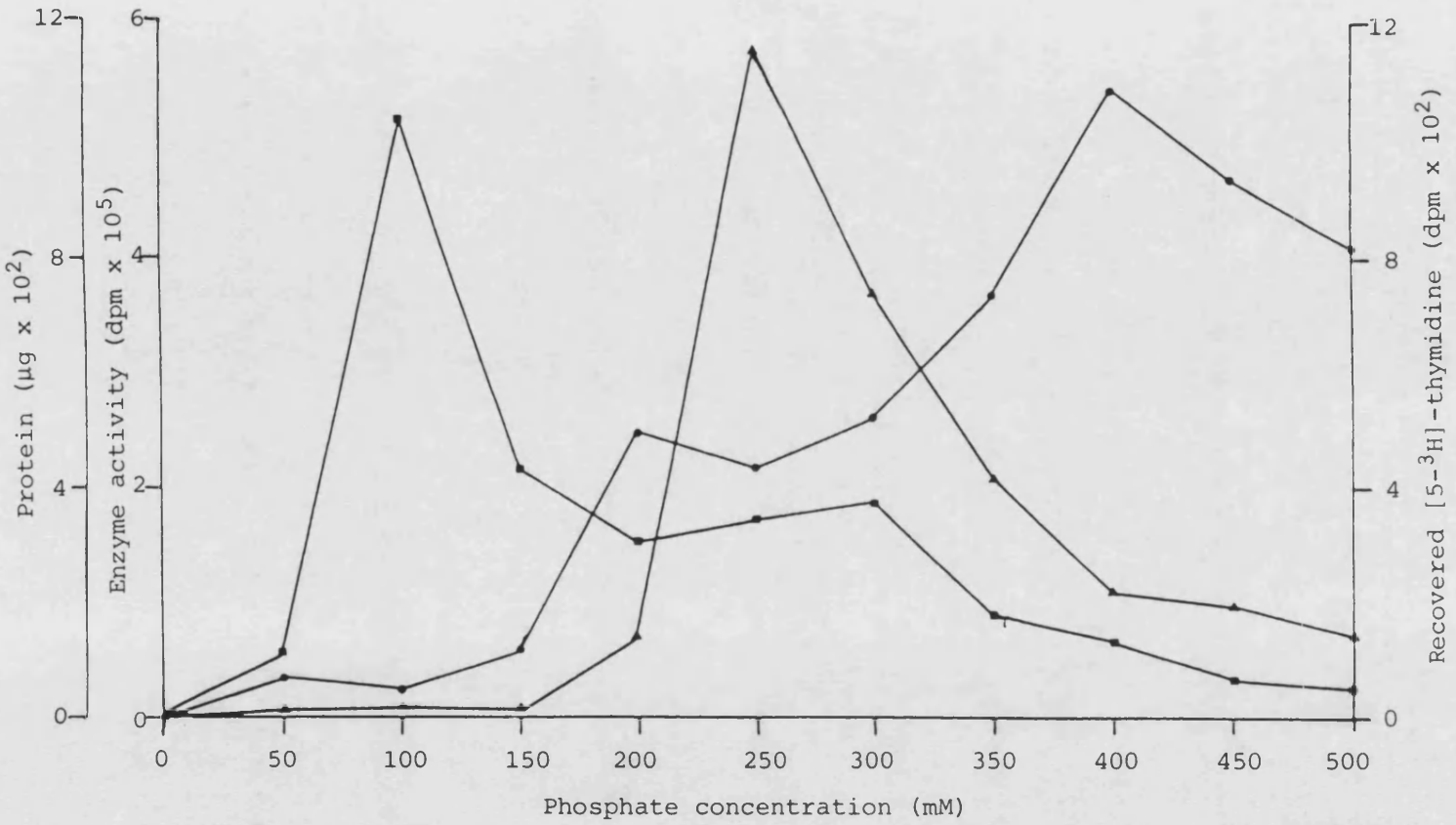


Figure 5 HA column chromatography of combined murine L1210 and pig thymus nuclear extracts.

0.4 µCi L1210 extract (section 3.2.1) and 2 ml nuclear extract were loaded onto a 2 ml HA column and stepwise elution carried out with increasing concentrations of potassium phosphate buffers.

All fractions (2 ml) were assayed for enzyme activity (●), protein (▲) and DNA (■).

specific activity of 4183 dpm/µg. This was a 4.8 fold increase on the specific activity of the crude extract.

Protein recovery was approximately 100% with maximum elution occurring at 100 mM followed by a second smaller peak at 300 mM.

34% of bound DNA was recovered from the column and the enzyme rich fractions (350-500 mM) contained 10% of the total DNA.

3.3.5 Two step chromatography using BioGel TE-2 and HA

0.4 µCi L1210 extract was mixed with 2 ml nuclear extract and diluted to 20 ml. The mixture was transferred to a tube containing 2 ml settled BioGel TE-2 and the contents mixed on ice for 15 min. The gel was then pelleted and the supernatant poured over a 2 ml HA column. The column was washed with 10 ml of buffer and elution attempted as previously with phosphate buffer (Figure 6).

Behaviour of DNA in the system

Of 8.3×10^5 dpm present in the extract, 98% was retained on the ion exchange resin. The label which did not bind to the BioGel TE-2 was bound by the HA and of this, 72% was recovered. Half of the recovered DNA was present in the richest enzyme fraction at 300 mM phosphate.

Behaviour of ADPRT

50% of the applied activity bound to the BioGel TE-2, which was very high in relation to previous experiments. Of the unbound activity, 72% was retained by the HA column, all of which was recovered in the phosphate elution steps. Peak enzyme elution occurred at 350 mM phosphate with 38% of enzyme recovered.

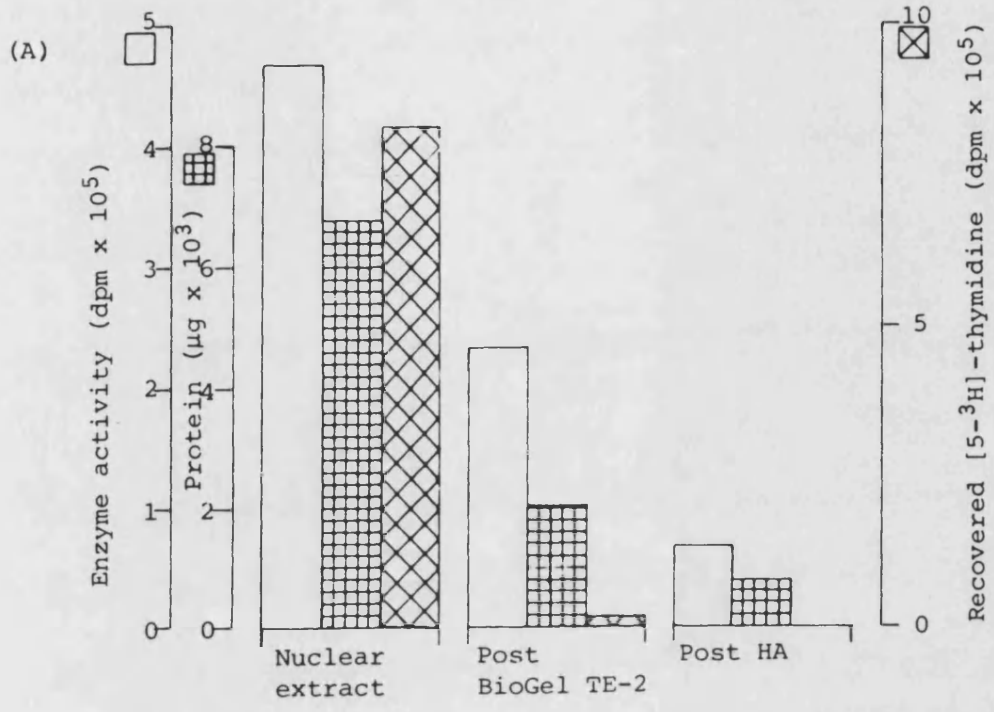
In the absence of DNA, all fractions were devoid of ADPRT activity, indicating that the residual DNA level was incapable of enzyme activation.

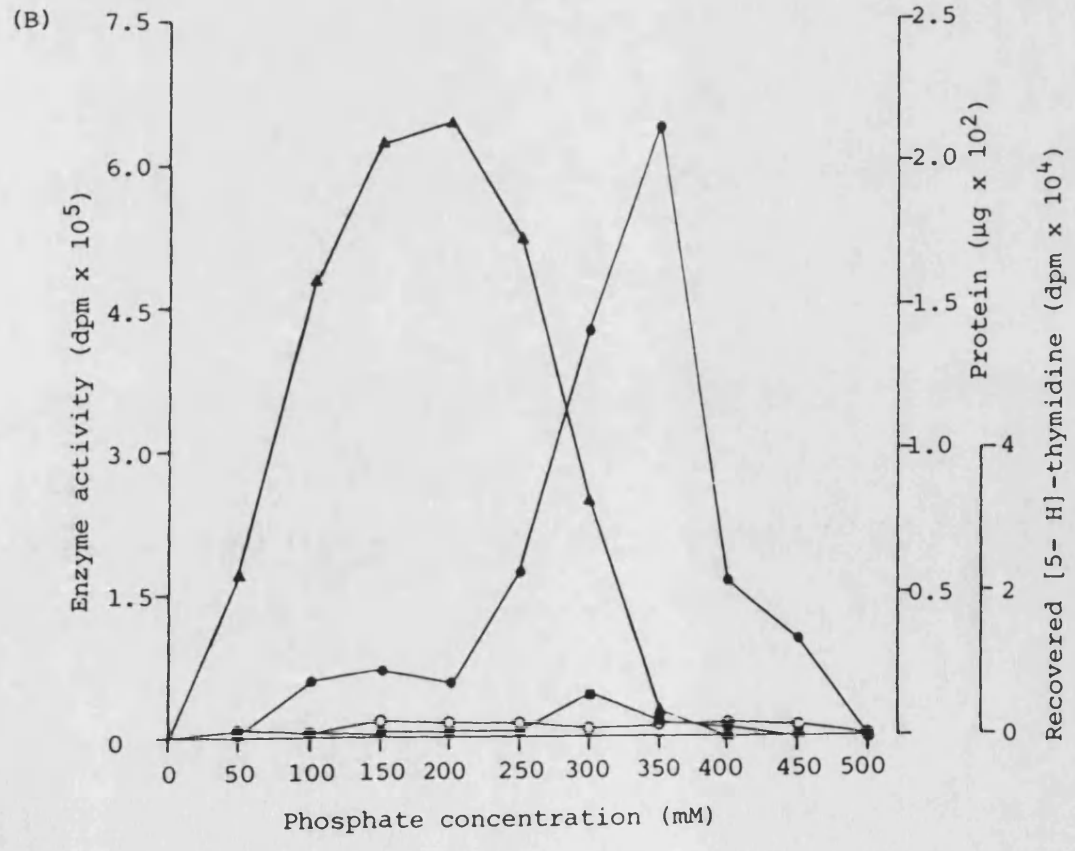
Figure 6 BioGel TE-2 and HA chromatography.

0.4 µCi L1210 extract (section 3.2.1) was combined with 2 ml nuclear extract and chromatographed as described in the text (section 3.3.5).

(A) Profile of nuclear extract before and after BioGel TE-2 and HA treatment.

(B) Stepwise HA elution profile. Each fraction (2 ml) was assayed for enzyme activity in the presence (●) and absence (○) of DNA, Protein (▲) and DNA (■).


(A)
Enzyme activity (dpm x 10^5)
Protein (µg x 10^3)
Recovered [5-^{3}H]-thymidine (dpm x 10^5)
Nuclear extract
Post BioGel TE-2
Post HA


(B)
Enzyme activity (dpm x 10^5)
Protein (µg x 10^2)
Recovered [5- H]-thymidine (dpm x 10^4)
Phosphate concentration (mM)

Behaviour of protein in the system

69% of the extract protein was retained by the ion exchange resin, 60% of the remaining protein bound to HA with 71% of the bound material recovered overall by phosphate elution. Peak protein recovery was observed with 200 mM phosphate.

Although approximately half of the enzyme activity was sacrificed in the ion exchange step, this was considered a justifiable loss when contrasted with the successful removal of DNA.

3.3.6 Comparison of ion exchange supports, Cellex T, Cellex QAE and BioGel TE-2

At this point, a suitable replacement had to be found for BioGel TE-2, as it was discovered that BioRad laboratories had discontinued production.

The anion exchangers Cellex T and Cellex QAE were obtained from BioRad laboratories.

Cellex T pK ∿9.5

$$O\text{-}C_2H_4\text{-}N^+\text{-}(C_2H_4)_3.Br^-$$

Cellex QAE pK - strongly basic

$$O\text{-}C_2H_4\text{-}N^+\text{-}(C_2H_5)_2\text{-}CH_2CHOH\ CH_3.Br^-$$

BioGel TE-2

$$O\text{-}CH(OH)\text{-}CH_2\text{-}N^+\text{-}(CH_3)_3.Cl^-$$

1 g of each support was prepared according to the manufacturer's instructions and equilibrated in 0.1 M TEA.HCl pH 8.0, 10 mM $MgCl_2$, 5 mM β-ME. 1 ml of each adsorbant was mixed with 5 ml diluted

nuclear extract and after mixing for 15 min at 0 °C, the resin pelleted. Each supernatant was assayed for enzyme and protein (Figure 7).

Unfortunately BioGel TE-2 gave the best purification. 54% of activity was not retained with the specific activity of the supernatant being 908 dpm/µg protein.

72% of enzyme activity bound to Cellex T resulted in a supernatant specific activity of 464 dpm/µg. The loss of activity observed was considered unacceptable. The highly basic adsorbant, Cellex QAE, was more hopeful, with little difference in the retention of activity. The supernatant had a lower specific activity than the crude extract (634 dpm/µg compared to 782 dpm/µg).

The positive charge held by Cellex QAE proved equally suitable for L1210 DNA removal in an identical experiment (result not shown).

3.4 Scaling up of Purification Procedures

In order to carry out protein characterisation studies and raise antibodies against the enzyme, the nuclei extraction and HA chromatography steps had to be scaled up to generate enough material for further processing.

Initially salt extraction, followed by ultracentrifugation in an SW-28 rotor overnight, was attempted. This raised the volume to 100 ml per preparation while maintaining similar activity levels. Although proving suitable for semi-preparative developmental work, the total amount of enzyme present was still restrictive.

A method for processing 200 g pig thymus tissue at a time was therefore developed.

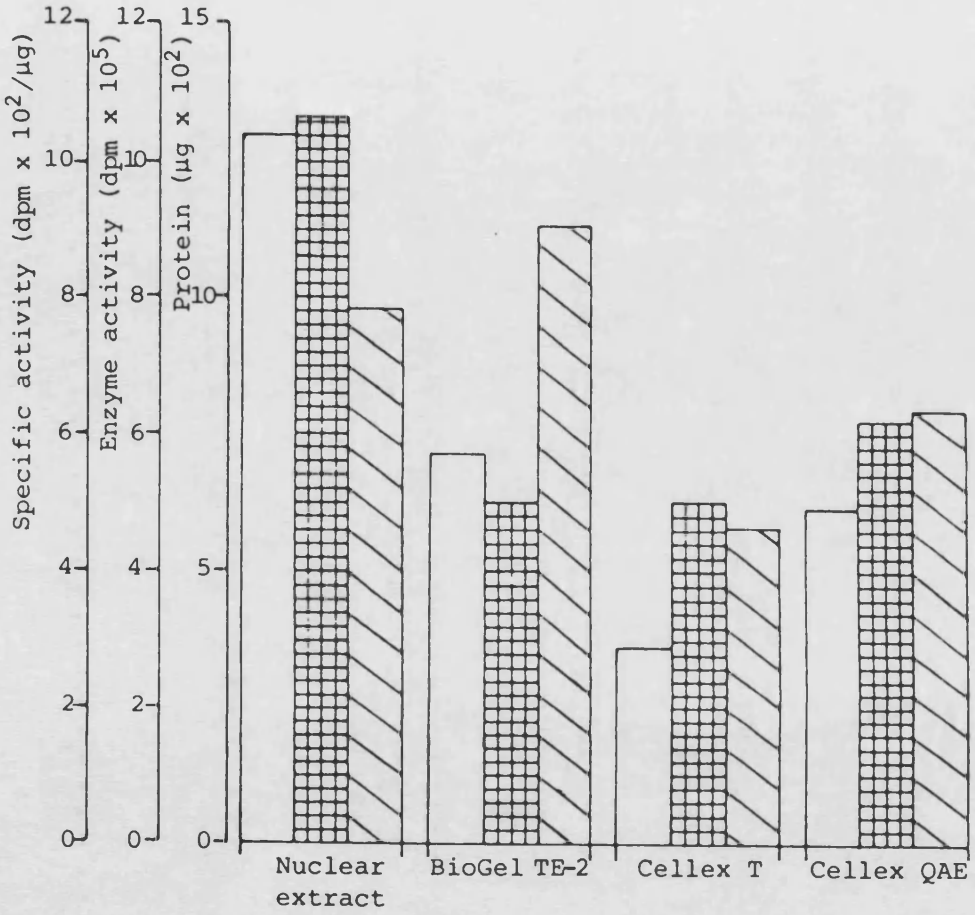


Figure 7 Comparison of anion exchange supports.

1 ml of each swollen adsorbant was mixed with 5 ml diluted nuclear extract. Following centrifugation, the enzyme activity (▭) and protein content (▦) of each supernatant was determined and used to calculate the specific activities (▨).

3.4.1 Extraction procedure for 200 g thymus tissue

Four 50 mg portions of frozen thymus tissue were individually homogenised (as described in Section 3.1) in 500 ml Tris.HCl pH 7.5, 0.32 M sucrose, 3 mM $MgCl_2$, 5 mM β-ME. Each homogenate was filtered through one layer of muslin and the nuclei pelleted by centrifugation at 5000 rpm for 20 min at 4 °C in an MSE 18 centrifuge.

The nuclei were resuspended in 4 x 180 ml volumes of ice cold 20 mM Tris.HCl pH 7.5, 0.15 M NaCl, 5 mM β-ME and pooled. 130 ml aliquots of suspension were then transferred to 300 ml centrifuge tubes and 130 ml salt buffer added (TEA.HCl pH 8.0, 1 M NaCl, 10 mM $MgCl_2$, 5 mM β-ME). The tubes were capped and mixed well by inversion, followed by centrifugation at 4 °C for 3 h in a Sorvall RC-5B machine at 13000 rpm. Typically extract of similar activity in the order of 750 to 800 ml was obtained, although the supernatant noticeably contained higher levels of DNA as judged by its viscosity.

3.4.2 Large scale batch chromatography using Cellex QAE and HA

Large scale DNA removal was carried out in batches using quantities of Cellex QAE calculated from the aforementioned [^{3}H-T] labelled L1210 extract studies. The extract, typically 500 ml, was diluted to 5 litres and mixed with 50 ml, packed volume, Cellex QAE. After stirring for 30 min at 4 °C, the extract was vacuum filtered through Whatman No.54 filter paper and the filtrate added to 50 ml, packed volume, HA. After a similar stirring period, the HA was allowed to settle out and the supernatant discarded. The remaining slurry was transferred to a glass chromatography column, the settled bed washed and elution carried out as previously with aliquots (50 ml) of increasing concentrations of phosphate (Figure 8).

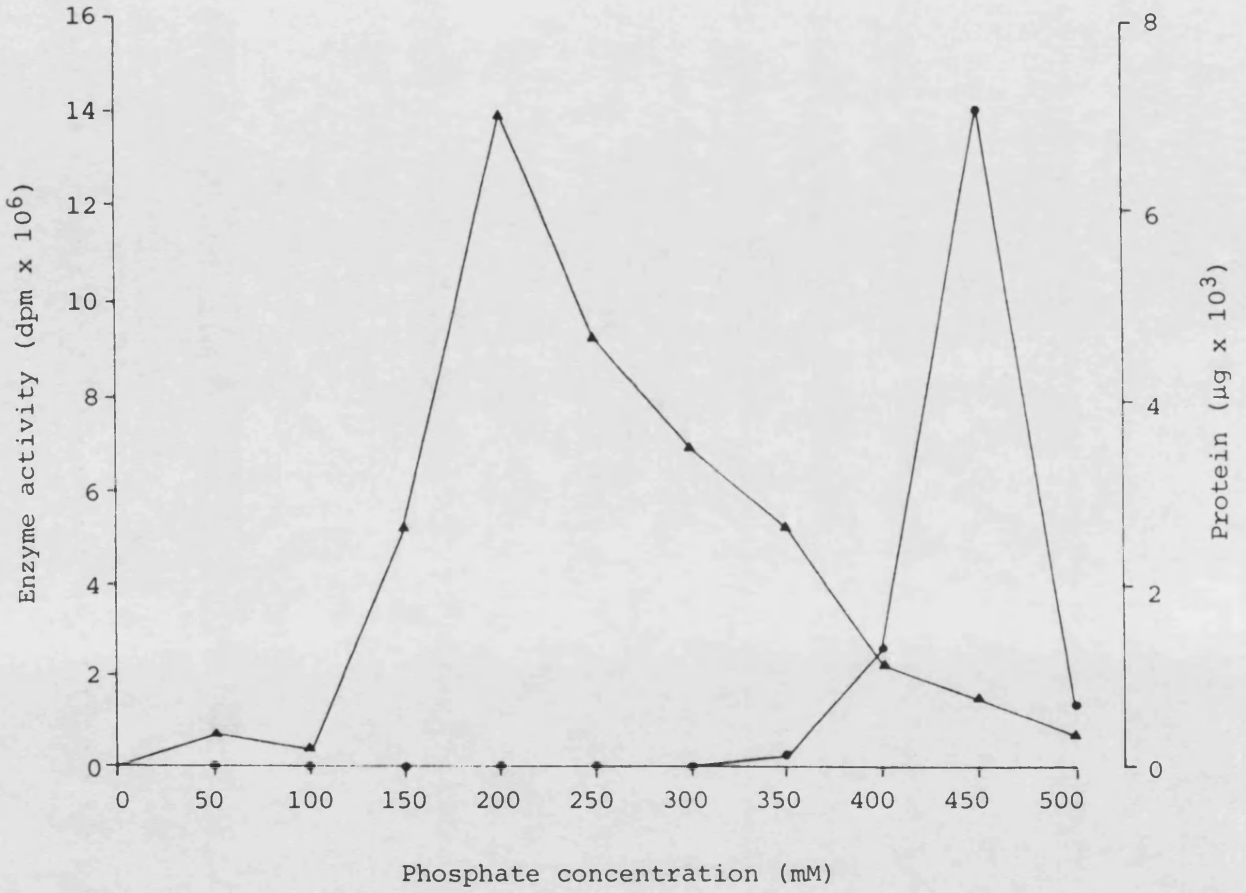


Figure 8 Large scale HA chromatography.

500 ml nuclear extract was processed with Cellex QAE (section 3.4.2) and mixed with 50 ml, packed volume, HA. The adsorbant was settled out, transferred to a glass column and elution carried out stepwise with 50 ml volumes of potassium phosphate buffers.

Each fraction was assayed for enzyme activity (●) and protein content (▲).

SDS-PAGE analysis of the enzyme rich fractions, using the Laemmli system, showed that the HA purified material was still very heterogeneous in nature. (The result is not shown due to destruction of the negative.) This procedure, however, was far from ideal for a number of reasons, excluding problems faced by handling 5 litres of extract.

It became increasingly apparent that the Cellex QAE step was unsatisfactory. Apart from continual loss of adsorbant through irreversible binding of anionic material, enzyme/matrix interaction was also very inconsistent and difficult to control. Enzyme binding occasionally varied by as much as one order of magnitude, although this was partly due to variable proteolysis. Addition of sodium bisulphite (25 mM) throughout extraction reduced the degree of apparent fluctuation significantly, but not to acceptable levels.

Stirring of the suspensions also proved critical. When agitation was too violent, particularly with HA, the adsorbant was irreversibly damaged, leading to the generation of very small sized fines. Filtration proved prohibitively time consuming as a result and settling of HA proved the only alternative. Unfortunately, the large surface area offered by the fine particles accounted for significant losses in enzyme activity when the fines were discarded.

In the long run, continual losses of Cellex QAE and HA would have proved unjustifiably costly. It was subsequently very important to find a reliable means of DNA removal from the extract.

3.5 Use of Polyethyleneimine (PEI) as a DNA Precipitating Agent

3.5.1 Effect of Polymin P on ADPRT activity

A number of protocols for the purification of chromatin bound enzymes has utilised addition of basic macromomolecules successfully to precipitate interfering DNA residues from crude extracts. As yield of enzyme and removal of DNA had been to date very inconsistent, PEI was tested for its ability to precipitate such contamination from the nuclear extract. The pKa of the imine group is about pH 6.5 and hence carried a positive charge at pH 7.5 on 10% of the residues. Such a charge allows interaction with the dissociated phosphate residues on the DNA backbone.

Initial study

A 1:4 solution of Polymin P (BDH - supplied as a 50% PEI v/v syrup) in distilled H_2O and a 1 M NaCl solution in 0.1 M TEA.HCl pH 8.0 were used for all Polymin and salt additions unless otherwise stated. Three microfuge tubes were set up, each containing 0.5 ml fresh nuclei. To tube 1 was added 0.5 ml salt buffer and to tubes 2 and 3, 10 and 50 µl of Polymin respectively. A fourth tube was also set up containing 0.5 ml buffer, 0.5 ml salt and 50 µl Polymin. The tubes were mixed well and allowed to stand on ice for 10 min. 0.5 ml salt was then added to tubes 2 and 3, followed by mixing and a further 10 min incubation on ice. All of the tubes were then microfuged at high speed for 2 min, the volume of supernatant noted and initial enzyme activities of the supernatants obtained, using the acid washed disc method (40 µg DNA was added to each assay).

Tube No.	Supernatant volume (%)	Initial rate (dpm/min)	Total recovery (dpm x 10^5/min/ml)
1	5	3600	4.37
2	95	2528	58.24
3	95	185	4.26
4	100	0	0

Surprisingly, addition of Polymin, prior to salt extraction of whole nuclei, to a concentration of 0.1% (v/v), proved very efficient in releasing enzyme from chromatin and dramatically reducing the pellet volume (approximately 5%).

0.5% Polymin treated nuclei showed enzyme activity, less than 10% of that observed with the lower concentration. This was explained, however, by excess Polymin binding the added DNA in the assay mixture, thereby minimising the activating effect on the enzyme. Tube 4, designed to quantify any non-enzymic contributions, showed no apparent increase in dpm over background.

The very promising results prompted further study.

3.5.2 Optimisation of enzyme dissociation time

To ensure that the 10 min time period was sufficient to allow maximum enzyme recovery, the following experiment was carried out.

5 tubes were set up, each containing 0.5 ml nuclear suspension and 10 µl Polymin. 0.5 ml salt buffer was added to each tube and the tubes mixed, followed by incubation for varying time periods on ice. At the appropriate times, the tubes were spun for 2 min in a microfuge at high speed and the supernatants assayed for enzyme activity. A sixth tube containing 0.5 ml nuclei was mixed with 0.5 ml salt, spun for 2 min, and its supernatant also assayed. The supernatant volume was approximately 85% in the Polymin tubes and approximately 5% in the salt extraction only tube.

Tube	Initial rate (dpm/min x 10^3)	Total recovery (dpm x 10^6/min/ml nuclei)
10 min	4.23	8.72
20 min	3.43	7.07
30 min	3.78	7.79
45 min	3.40	7.01
60 min	4.17	8.60
Non-Polymin extract	3.40	0.41

10 min extraction time allowed recovery of enzyme which could not be improved upon by increased incubation time. The amount of enzyme released was approximately the same at all incubation times, allowing for experimental error.

3.5.3 Optimisation of Polymin concentration

The next logical step was to determine whether the Polymin concentration of 0.1% v/v was optimal.

6 tubes were set up as previously, containing 0.5 ml nuclear suspension. 10 µl of a serial dilution of Polymin in distilled H_2O was added to each tube, mixed and incubated on ice for 10 min. 0.5 ml salt was then added, mixed, and after 10 min on ice, the tubes were microfuged and the supernatants assayed.

Polymin conc. (% x 10^{-3})	Supernatant vol. (%)	Initial rate (dpm/min)	Total recovery (dpm x 10^6/min/ml nuclei)
100	85	3267	6.73
50	45	2147	2.34
25	10	2050	0.50
12.5	<5	-	-
6.25	<5	-	-
3.125	<5	-	-

At a concentration of 100 x 10^{-3}%, the Polymin treated nuclei did not appear to be broken. As the percentage was reduced, however, the ability of the Polymin to hold together the nuclei after addition of salt, was reduced accordingly. Below 25 x 10^{-3}%, the addition of Polymin had no noticeable effect on the nuclei under conditions of high salt and the volumes of the supernatants reflected this.

3.5.4 Optimisation of salt concentration

The next step was optimisation of the salt concentration required to dissociate enzyme from the nuclei.

5 microfuge tubes were set up, containing 0.5 ml nuclear suspension. 10 µl Polymin was added to each tube and incubation on ice carried out as before. The tubes were then mixed with 0.5 ml solutions of varying NaCl concentration in TEA.HCl pH 8.0, before incubation on ice and centrifugation in a microfuge as previously. All supernatant volumes were measured and assayed for ADPRT activity and protein content.

An ordinary salt extract was also prepared by mixing 10 ml nuclei with 10 ml salt solution. The mixture was centrifuged for 15 min at 15000 rpm (8 x 50 ml Sorvall) before the supernatant was taken and assayed as above.(see table overleaf).

Maximum recovery of activity was attained with a salt concentration of 0.4 M. At this concentration 45% more activity was recovered, per ml nuclei, than from the 'non-Polymined' salt extract. The results also indicated that this concentration represented the optimal concentration for protein dissociation overall, as maximum protein recovery was also observed at this level.

Sample	Supernatant volume (%)	Initial rate ($dpm \times 10^3/min$)	Recovery per ml nuclei ($dpm \times 10^6/min$)	Protein (mg/ml nuclei)	Specific activity ($dpm \times 10^6/min/mg$)
Non-Polymin extract	30	4.23	3.08	0.96	3.21
0.5 (M)	82.5	1.55	3.10	2.05	1.52
0.4 (M)	85	2.17	4.47	2.14	2.09
0.3 (M)	85	2.03	4.18	1.56	2.67
0.2 (M)	90	1.20	2.62	1.21	2.17
0.1 (M)	85	0.92	1.89	0.56	3.37

3.5.5 Scaling up of salt extraction of Polymin treated nuclei (from 12.5-200 g thymus tissue)

Nuclei were isolated as previously and resuspended in 500 ml TEA.HCl pH 8.0, giving a total volume of 570 ml. The suspension was divided into three portions, which were treated as follows:-

(1) 10 ml were mixed with 10 ml 1 M salt buffer, incubated on ice for 10 min, and centrifuged at 4 °C for 15 min at 15000 rpm (Sorvall 8 x 50 ml head).

(2) 280 ml were mixed very slowly with 5 ml Polymin (over 2 min). After a 10 min incubation on ice, the suspension was divided equally into 3 Sorvall tubes (250 ml) and each tube mixed well with 93 ml 0.8 M salt in TEA.HCl pH 8.0. After a further 10 min on ice, the contents were centrifuged at 4 °C for 15 min at 12.5 krpm.

(3) 280 ml were treated as in (2) above, except 93 ml of an 0.6 M salt solution was mixed with the contents of each tube.

After centrifugation, the supernatants were assayed for ADPRT activity and protein content (see table overleaf). In the previous small scale experiment, 0.4 M salt gave optimal enzyme activity recovery. When scaled up as described, however, 0.3 M salt extraction gave a better recovery than 0.4 M. The enzyme activities recovered, per ml of nuclei extracted, were 4.2 and 3.0 times the activity recovered from 0.5 M salt extraction in the absence of Polymin, with 0.3 M and 0.4 M salt respectively. The specific

Sample	Volume Total (ml)	Supernatant Volume (%)	Initial rate (dpm x 10^3/min)	Recovery per ml nuclei (dpm x 10^6/min)	Protein (mg/ml nuclei)	Specific activity (dpm x 10^6/min/mg)
Non-Polymin extract	20	20	4.55	2.21	0.53	4.15
0.4 (M)	584	92	2.95	6.57	2.32	2.83
0.3 (M)	584	96	3.96	9.22	2.09	4.40

activity of the 0.3 M extract was also higher than the Polymin free extract. 0.3 M salt was therefore used for all further extractions.

3.5.6 Binding of Polymin treated extract to HA

Once the PEI step had been optimised, the next factors to determine were the dependency of the extract on DNA for activity and the possible effect on binding to HA.

A 0.5 ml column was poured and equilibrated with 5 mM potassium phosphate pH 8.0, 5 mM β-ME, 25 mM $Na_2S_2O_5$. 5 ml nuclear extract was passed through the column followed by a 5 ml wash with the same buffer. The extract was assayed, before and after loading, in the presence and absence of DNA.

Sample	+DNA (dpm x 10^3)	-DNA (dpm x 10^3)	(%)
Nuclear extract	2696	18	0.7
Post HA + wash	40	0	0

99.3% of activity was lost on omission of DNA from the assay, indicating that the extract was essentially DNA free. 98.5% of enzyme activity bound to the column. A large scale binding and elution experiment was therefore set up.

3.5.7 Large scale HA chromatography of Polymin treated salt extract

1080 ml extract was loaded onto a 25 ml HA column (2.5 x 5.1 cm) equilibrated with 0.1 M TEA.HCl pH 8.0, 5 mM β-ME, 25 mM $Na_2S_2O_5$. The loaded column was washed with 50 ml 5 mM phosphate pH 8.0; 5 mM β-ME; 300 mM KCl, 25 mM $Na_2S_2O_5$. The washed column was then eluted with 300 ml of a linear potassium phosphate gradient from 5 to 600 mM. The flow rate was maintained throughout at 50 cm/h and all fractions (12 ml each) were assayed for protein

and enzyme activity in the presence and absence of DNA. (No impairment of flow rate was observed throughout the separation.)

As can be seen in Figure 9, excellent separation was achieved, 70% of applied enzyme and 43% of the protein was retained by the column. 97% of the bound enzyme was recovered in a bell shaped peak from approximately 220 to 455 mM phosphate with maximum recovery at a concentration of 360 mM. Peak protein elution was observed at 100 mM. Comparison of the specific activity of fraction 15 from the column (19×10^6 dpm/mg) with the pre column extract (1.56×10^6 dpm/mg) gave a maximal purification of greater than 12 in this step.

As in the previous experiment, the extract, as well as the column fractions, were dependent on addition of exogenous DNA to the assays for activity. The fractions containing activity assayed in the absence of DNA were those eluted slightly after the fractions containing maximum activity assayed in the presence of added DNA. The observed peak of enzyme activity shifted from 360 mM in the presence of DNA, to 410 mM phosphate in the absence of DNA. This is probably explained by co-elution of trace amounts of double stranded DNA fragments not removed by the Polymin step.

The suitability of the PEI treated extract for immediate HA column chromatography, coupled with the speed and very high recoveries of activity (>95% recorded in most cases) from HA, culminated in a very significant advancement in the purification protocol.

SDS-PAGE analysis of the enzyme rich fraction 15 alongside nuclear extract also showed significant enrichment of a protein of approximately 116 Kd in size (Plate 1).

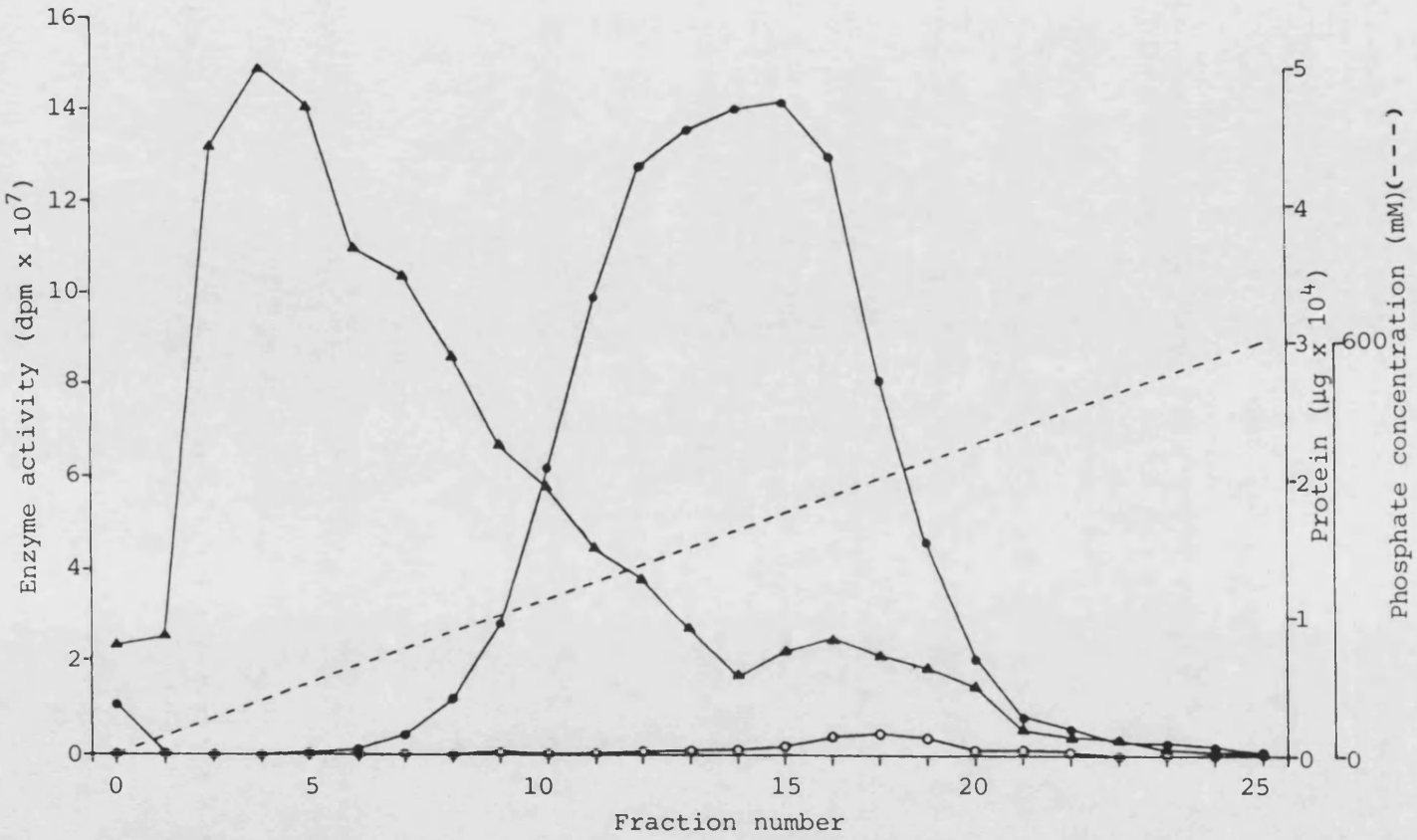


Figure 9 Large scale HA column chromatography of Polymin treated nuclear extract.

1080 ml nuclear extract was loaded directly onto a 25 ml HA column. The column was then washed and elution carried out with a 300 ml linear phosphate gradient (5-600 mM).

Each fraction (12 ml) was assayed for protein content (▲) and enzyme activity in the presence (●) and absence (O) of DNA.

PLATE 1 SDS-PAGE analysis of Polymin treated nuclear extract and HA enriched ADPRT extract

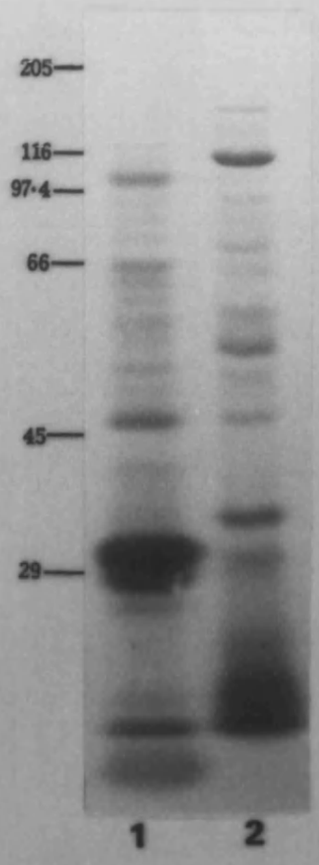


Plate 1 SDS-PAGE analysis of Polymin treated nuclear extract [1] and HA enriched ADPRT extract (Figure 9, fraction 15) [2] using the Laemmli buffer system. The equivalent of 50 µg of each sample was loaded alongside the following molecular weight markers [MW-SDS-200-(Sigma)]: Myosin (205 Kd), β-Galactosidase (116 Kd), Phosphorylase b (97.4 Kd), Bovine albumin (66 Kd), Egg albumin (45 Kd), Carbonic anhydrase (29 Kd).

Column overloading

The large amount of unretained material probably arose from column overloading. Recirculation could have been utilised, but would have required vastly longer application times, besides which, the column size could quite easily be increased without altering the nature of the separation.

All future separations were carried out on a 50 ml HA column, with the result that retention was always more than 95% of the activity applied.

3.6 Gel Filtration Chromatography

Also called gel permeation chromatography, this method is a separation technique based primarily on size differences. Acrylamide or highly hydrated carbohydrate polymer beads are used as the separation medium. The porous nature of the beads is such that their interiors are accessible only to components of the mixture below certain controlled size limitations. Such a limitation is termed the exclusion limit of that particular matrix and will be determined by the nature of the separation required. Components of the mixture above the exclusion limit pass straight through the column emerging first. Included components emerge later than excluded components in order of decreasing size.

Chromatography of HA purified ADPRT

Study of the distribution of proteins in the enzyme rich fractions eluted from large scale HA on 7.5% w/v SDS-polyacrylamide gels showed a significant amount of material below 100 Kd in size. Chromatography of the ADPRT rich HA fractions in a matrix

with an exclusion limit of 100 Kd was attempted as an enrichment step. The matrix chosen was BioGel P-100, an inert polyacrylamide medium (BioRad).

A 53 ml column (30 x 1.5 cm) was poured and equilibrated with reservoir buffer: 50 mM potassium phosphate pH 8.0, 5 mM β-ME, 20 mM KCl, 25 mM $Na_2S_2O_5$ (KCl minimises ionic interactions caused by small quantities of carboxyl groups present on the medium).

Void volume determination

0.5 ml of a solution of 0.3% w/v Blue dextran (BDH), 20 mM KCl, 1% w/v sucrose, was layered onto the top of the gel and allowed to pass through the column at a constant linear flow rate of 5 cm/h. The high molecular weight of the Blue dextran (>2 x 10^6 d) causes exclusion from the gel. Buffer is collected from the column outlet until the dextran emerges and the total volume collected at this point is recorded as the excluded or void volume.

3.5.1 Chromatography of HA purified ADPRT

10 ml enzyme rich HA material was reduced to 2 ml by ultrafiltration and the extract supplemented with 10% v/v glycerol. The column flow was stopped, the buffer reservoir disconnected and the enzyme extract layered onto the top of the gel. The buffer was reconnected, flow resumed, and the flow rate maintained at 5 cm/h. 1 ml fractions were collected and assayed for enzyme activity and protein content (see Figure 10).

Gel filtration at this stage was of limited use, however. The large time scales involved in sample concentration and the flow rates involved with gel filtration generally would prove

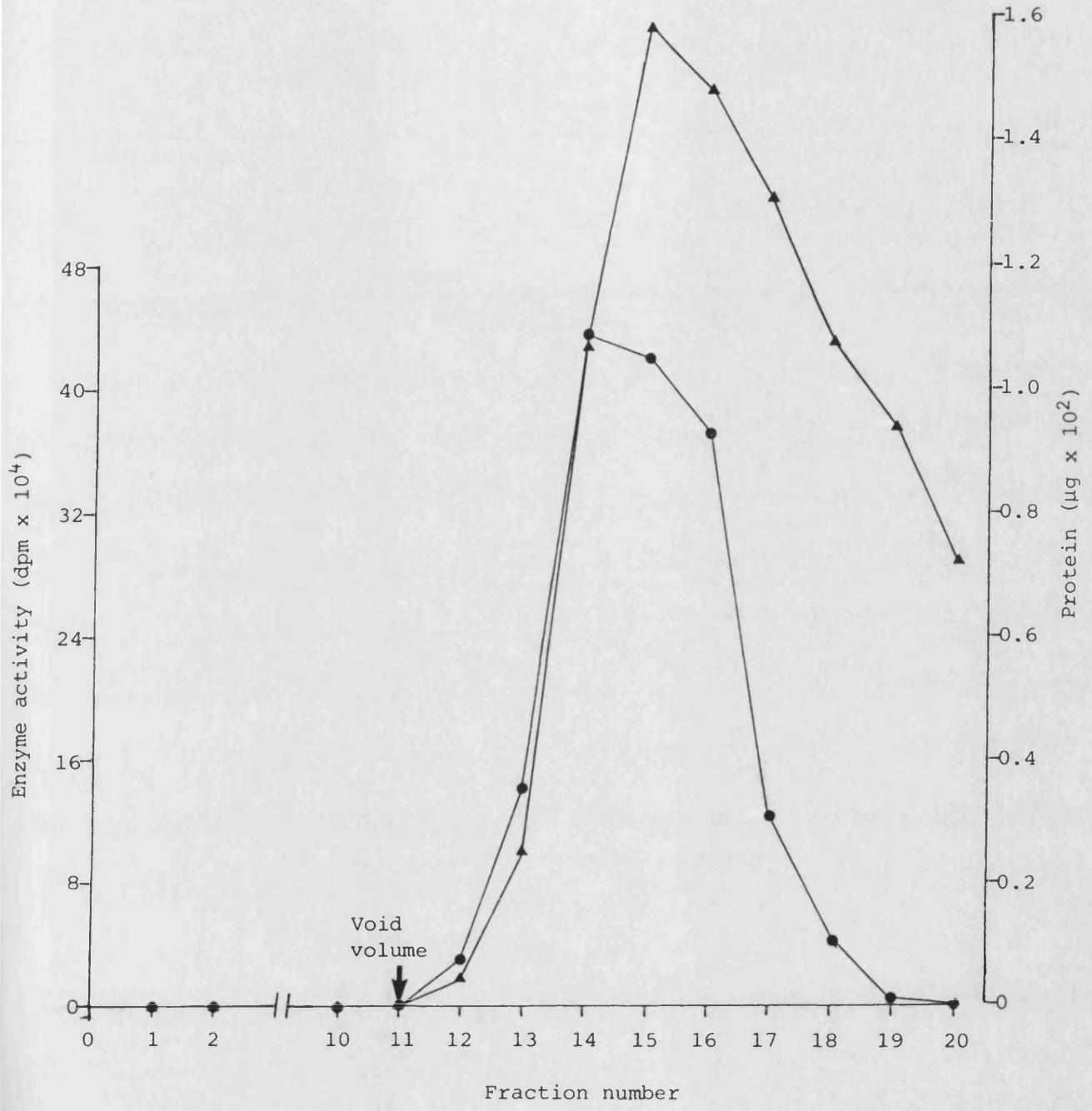


Figure 10 BioGel P-100 chromatography of HA purified ADPRT.

2 ml concentrated HA purified material was chromatographed on a 30 x 1.5 cm column at a constant flow rate of 5 cm/h (section 3.5.1).

All fractions (1 ml) were assayed for enzyme activity (●) and protein (▲).

restrictive with large scale purifications. An overall purification factor of 2.2 fold was achieved.

Gel filtration is more usually used as a final clean up step in purification protocols to remove minor amounts of contaminating species of varying sizes. For such separations, a gel is chosen such that the protein of interest is included in the matrix (assuming contaminants are both smaller and larger than the desired species).

The technique was discarded at this stage, but was, indeed, tried as a final step in the developed protocol (see later).

CHAPTER 4

Affinity Chromatography

4.1 Introduction

This is a powerful technique used widely in the purification of a whole host of biological molecules (Wilchek *et al.*, 1984). The technique exploits the specific reversible interaction of proteins with a ligand immobilised on an insoluble support. Substrates or their analogues, or antibodies and antigens are typical choices as ligands.

The protein mixture containing the molecule of interest is passed through such a column containing immobilised ligand, allowing specific recognition and binding to take place (Cuatrecasas *et al.*, 1968). The components of the mixture with no significant affinity are removed by washing prior to elution of the bound material.

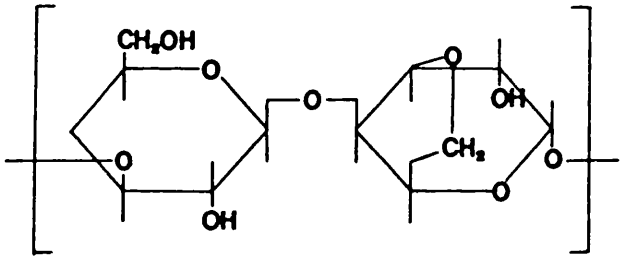
Affinity chromatography of enzymes is usually either mono-specific, utilising competitive inhibitors, substrates, cofactors and coenzymes as immobilised ligand, or group specific, involving use of ligand common to a whole host of enzymes - NAD, ATP and CoA being such examples.

Choice of matrix

The choice of insoluble support for affinity chromatography is quite critical. The material must be biologically inert, capable of derivatisation under mild conditions, of reasonable rigidity, of regular bead and pore size to allow good flow properties, chemically stable to binding and elution conditions and of moderate hydrophilicity (Wilchek *et al.*, 1984).

Agarose

To date, agarose is the most popular choice. It was initially introduced as a gel filtration medium (Hjertén, 1962a) and has been used extensively since. The material, an essentially uncharged component of agar, is a polysaccharide composed of the repeating "agarobiose" unit:



(Porath, 1968)

(Porath, 1968)

There is no covalent cross-linking in the gel, hydrogen-bonding being responsible for the tertiary structure. The disadvantages associated with agarose largely stem from this fact. In particular, thermal instability, shrinkage, solubility in some chaotropes, and its incompatibility with certain organic solvents can cause difficulties. Cross-linking with epichlorhydrin may reduce such problems, although a proportion of the free hydroxyl groups are utilised resulting in decreased ligand capacity.

Agarose notably has a very low fixed charge group content reducing ionic interactions to a minimum. Carboxyl and sulphate groups are evident in most commercial preparations, but only in very low levels. Indeed, sulphur content is usually used to assess purity, with up to 0.37% found in the majority of commercially available material.

Agarose gel exhibits far greater rigidity and porosity than comparable polyacrylamide and carbohydrate gels of similar concentrations. Only below 2% do gels exhibit significant degrees of compacting under chromatography conditions.

As a result of the above properties, the medium ranks as an almost ideal choice for most affinity chromatography requirements.

Polyacrylamide

Bead forms of polyacrylamide have also been used in affinity chromatography (Hjertén, 1962b; Inman and Dintzis, 1969). The gel beads are composed of acrylamide polymer cross-linked with methylene-bisacrylamide. The nature of the gel can be strictly controlled by variation of the concentration of monomer and of the degree of cross-linking introduced.

Its principle advantages are the high number of chemically modifiable groups, allowing high levels of substitution, and the high degree of inertness exhibited by the column (Cuatrecasas, 1970).

Disadvantages include hydrolysis of carboxamide groups to carboxyls above pH 10 and the low degree of porosity observed in derivatised beads. Beads of only the highest porosity (BioGel P-300) were successful in the purification of staphylococcal nuclease (mol wt. 7 Kd) (Cuatrecasas, 1970). Unfortunately such beads are so soft that mechanical problems are almost inevitable.

Dextran derivatives

Cross-linked dextran gels were introduced in 1959 by Porath and Flodin. Like agarose, they found the material highly inert, although unfortunately it has proved of limited value since, due to the low porosity of the medium, particularly after derivatisation, in the mechanically stable forms.

Cellulose

Also of limited use to date, cellulose has proved far from ideal as a support due primarily to the non-spherical, non-porous nature of the medium. These factors, coupled with irregular particle size, do not contribute to a matrix which exhibits good flow properties.

Others

"Ultrogels" have far greater rigidity than most media, being composed of cross-linked polyacrylamide with interstitial agarose, and have the advantage of carrying two types of modifiable group (*i.e.*, hydroxyl and carboxamide).

Trisacryl gels are pronouncedly hydrophilic and should see beneficial use in high performance systems, due to their ability to withstand quite high pressures.

Polystyrene is another organic support that has been used, while inorganic examples include controlled pore glass, silica gel and alumina.

Elution of bound species

Recovery of bound material may be carried out specifically or non-specifically.

Specific elution is usually achieved by use of solutions of free ligands. Such ligands need not necessarily be identical to the immobilised species and may be substrates, inhibitors, co-factors, dyes or any other allosteric modifier of the bound species.

A large number of non-specific methods of elution have been used successfully. Popular methods such as pH and ionic strength manipulation are often effective, as well as being cheaper than

a number of specific methods. While such approaches alter the contribution made to binding by ionic interactions, perturbations in hydrophobic binding may also occur (Cuatrecasas, 1968). Such manipulations may also be used prior to specific elution methods in order to alter binding constants where very strong interactions are evident. It is worth noting, however, that decreased affinity of bound enzyme for free ligand may also result.

Temperature alterations can also have pronounced effects on protein-ligand binding, particularly where hydrophobicity makes a significant contribution to the interactions. Lowered temperature results in a decrease in hydrophobic interactions which may not lead to successful elution in its own right, but may promote easier desorption by specific means.

Chaotropic agents such as thiocyanate, SDS, urea and guanidinium salts have all been used with some success (Angal and Dean, 1977), the major limiting factor being the degree of success in achieving renaturation of the eluted components. Such compounds are of particular use in the case of antibody-antigen complexes.

Electrophoresis is a mild dissociation method which has seen high recoveries when used in desorption of charged proteins (Morgan, 1978).

Other methods

Where binding is so tight that the above methods fail, chemical cleavage of the bound protein from the support *via* the spacer arm may be necessary. Dissociation of complexes is often more favourable in solution and may be a feasible alternative, providing that the cleavage step is mild enough to maintain functional integrity of the bound protein.

Ester linkages are quite easily cleaved in mild base (Brown *et al.*, 1979; Singh *et al.*, 1979) and vicinol hydroxyls and diazobonds may be cleaved by periodate and dithionite respectively (Cuatrecasas, 1970a).

A problem inherent in such methods is irreversible damage to the affinity matrix which may prove impracticable in terms of cost and time.

Reverse immuno-adsorption has been used, which has the major advantage of not requiring an elution step (Weare *et al.*, 1982). In such a procedure, immunoglobulins raised against all components of a mixture, except the desired species, are used as immobilised affinity ligands.

Finally changes of buffer or solvent, addition of specific metal ions, the use of deformers, *i.e.*, compounds conferring reversible conformational changes on protein surfaces, and use of polarity reducing compounds like ethylene glycol, have all been used with some success.

4.2 Immobilised Dye Chromatography

The reactive triazine dyes were originally developed for use in the textile industry by ICI in the 1950s, but have since been recognised as valuable ligands in affinity chromatography (Lowe and Pearson, 1984). A number of supports have been used for immobilisation, including Sephadex, cellulose and polyacrylamide, although for reasons discussed in the previous section, agarose is reported to be the most successful. Attachment of the dye takes place *via* hydroxyl groups on the support, or to an immobilised spacer molecule.

The dyes have a number of advantages over immobilised cofactors and substrates as affinity ligands. In addition to their ease of

binding, they exhibit high capacities for protein and resistance to enzymatic degradation.

Immobilised dye chromatography is strictly speaking a pseudo-affinity technique. The structures of the molecules are such that they exhibit hydrophobic and electrostatic properties such that protein-dye interactions may be due to a mixture of non-specific charge and hydrophobic factors, as well as specific factors.

Choice of dye for a particular purification is usually made following screening of a variety of dye matrices. Generally speaking, proteins seem to have higher degrees of interaction (low Kd) with dyes of higher λ_{max}. Subsequently, the tighter binding blue and green dyes and the intermediate red dyes are preferred.

Loading of columns is carried out at moderate buffer strength (<100 mM) and intermediate pH (pH 5.5-9). Elution may be achieved specifically or non-specifically, as previously outlined.

In immobilised dye chromatography, as in all affinity chromatography systems, selective binding and elution is nearly always attained empirically. No hard and fast rules apply and no firm predictions can be made regarding the behaviour of any particular protein in a defined system.

Matrex Gel Red A Chromatography

Both Cibacron blue F3GA and Procion Red H-3B have been used extensively in the purification of a whole host of pyridine nucleotide dependant enzymes. Similarly, Zahradka and Ebisuzaki (1984), used Matrex Gel Red A (Amicon), an azodye-agarose matrix, successfully for the purification of calf thymus ADPRT.

Matrex Gel Red A was first assessed batchwise for binding and elution of ADPRT rich HA material.

To each of 6 tubes containing 0.5 ml Matrex Gel Red A, pre-washed with standard phosphate buffer [50 mM potassium phosphate pH 8.0, 5 mM β-ME, 25 mM $Na_2S_2O_5$ (SPB)] was added 4 ml ADPRT rich HA material, prediluted to 50 mM phosphate. The tubes were mixed on ice over a period of 30 minutes, after which the gel was pelleted in a bench centrifuge, followed by a 5 ml wash with buffer and another centrifugation step. The supernatant was discarded and 2 ml aliquots of TEA.HCl pH 8.0, 5 mM β-ME, 25 mM $Na_2S_2O_5$ containing varying amounts of KCl were added to the tubes. The tubes were mixed over a 30 minute period to allow elution, and following a final spin, the supernatants were assayed for enzyme and protein (Figure 11).

All of the applied activity bound to the gel, 62% of which was eluted with 1.25 M KCl. Binding appeared to be relatively independant of charge interaction, with only 2.4% of enzyme activity eluting below 500 mM KCl. Maximum enrichment was achieved with 0.75 M KCl.

Column Matrex Gel Red A Chromatography

Attempts at column chromatography were prompted by the promising result using batchwise separation.

A 1 ml column was poured and equilibrated in SPB, 8 ml of HA purified material was loaded onto the column, followed by a 5 ml wash (0.25 M KCl in SPB). Elution of bound material was carried out stepwise with 2 ml aliquots of increasing KCl in SPB. All fractions were assayed for protein content and enzyme activity (Figure 12).

98.9% of applied activity bound the gel with 52.7% recovered. Peak elution occurred at a KCl concentration of 0.9 M, the fraction

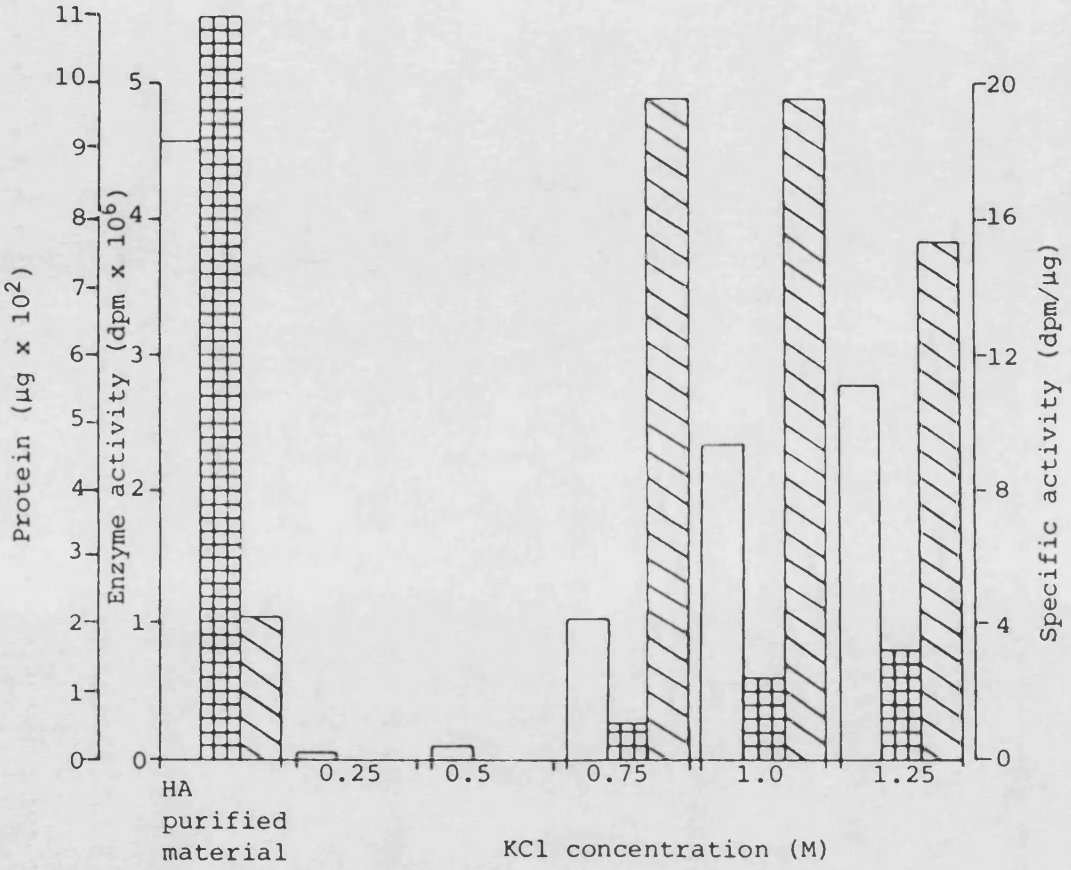


Figure 11 Matrex Gel Red A batch chromatography.

0.5 ml volumes of adsorbant were mixed with HA purified material and elution carried out with increasing concentrations of KCl.

Following centrifugation, the enzyme activity (□), and protein content (▦) of each supernatant was determined and used to calculate the specific activities (▨).

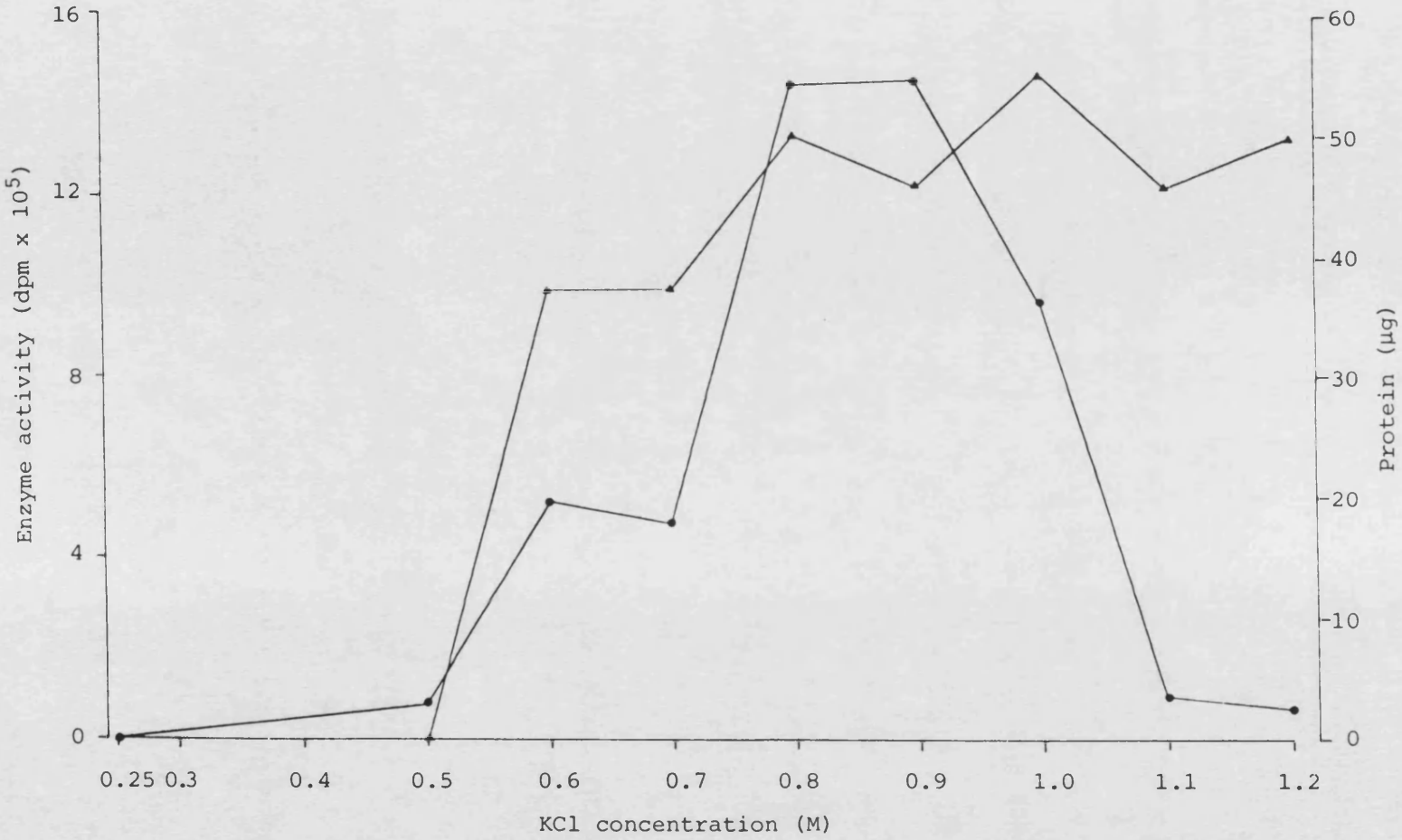


Figure 12 Matrex Gel Red A column chromatography.

8 ml HA purified material was chromatographed on a 1 ml column and elution carried out stepwise whth increasing concentrations of KCl.

All fractions were assayed for enzyme activity (●) and protein content (▲).

containing approximately 15% of the bound enzyme activity. 24% of bound protein was recovered.

Maximum purification was achieved at a KCl concentration of 0.9 M, representing a 4.3 fold enrichment. While acknowledging that this represented a significant purification, more specific affinity methods were pursued.

4.3 Chromatography using Benzamides as Affinity Ligands

The benzamide group of compounds recognised as ADPRT inhibitors (Section 1.3.5) have a number of features necessary for successful use as affinity ligands. Of particular value is their high degree of specificity for the enzyme and their low K_i values ($\sim 10^{-6}$ M). The compounds are stable to enzymic and chemical degradation at moderate pH and the nature of a number of substitution groups on the ring can facilitate easy covalent attachment to support matrices, either directly or *via* spacer molecules.

The major disadvantage associated with their use is their aromatic nature. Fortunately this is a serious problem only with high levels of column substitution. In such cases, the nature of the interactions involved switch from primarily specific to non-specific due to the increased hydrophobic nature of the column. The high affinity of the enzyme for these inhibitors necessitates only low levels of substitution, however, thereby minimising non-specific interactions.

4.3.1 Bisoxirane coupling of 3-hydroxybenzamide (3HB) to Sepharose-6B

Cyanogen bromide and bisoxiranes can be used successfully in the coupling of a large variety of amine and hydroxyl containing compounds to insoluble carbohydrate supports. The ether linkage resulting from the reaction of hydroxyl compounds with an oxirane or epoxide

group of a bisoxirane is extremely stable and therefore constitutes an ideal means for the attachment of the ADPRT inhibitor 3HB to Sepharose (Figure 13).

The activation step was carried out according to the method of Porath (1974). 5 ml of Sepharose-6B (Pharmacia) was used in the coupling step, which was washed extensively with distilled H_2O, followed by suction drying. The moist gel cake was then resuspended in 3 ml of 0.2 M sodium carbonate buffer pH 9.5 containing 500 µg 3HB, and the mixture, in a small conical flask, left shaking gently for 20 h at 25 °C. The coupled gel was then filtered and washed under suction with 500 ml distilled H_2O and 500 ml 1 M NaCl. The matrix was stored at 4 °C in 15% v/v ethanol.

4.3.2 Binding of ADPRT to the 3HB affinity matrix

A 1 ml column of affinity adsorbant was poured and equilibrated with SPB. 10 ml HA purified enzyme was loaded onto the column and reapplied continually for 1 h by means of a peristaltic pump.

Enzyme activity and protein content were assayed in triplicate before and after application.

3.3×10^6 dpm of the 4×10^6 dpm enzyme activity applied to the column was not retained, representing only 17% of the activity bound. 1.5 mg of protein was applied, of which 1.1 mg was recovered. The amount of retained enzyme was very low, whereas quite high quantities of protein bound. In view of the potency of the inhibitor employed, the result was disappointing.

Because of the susceptibility of the amide group to hydrolysis at high pH, relatively mild conditions to those recommended by Porath for hydroxyl compounds were used, *i.e.*, 25 °C at pH 9.5, instead of

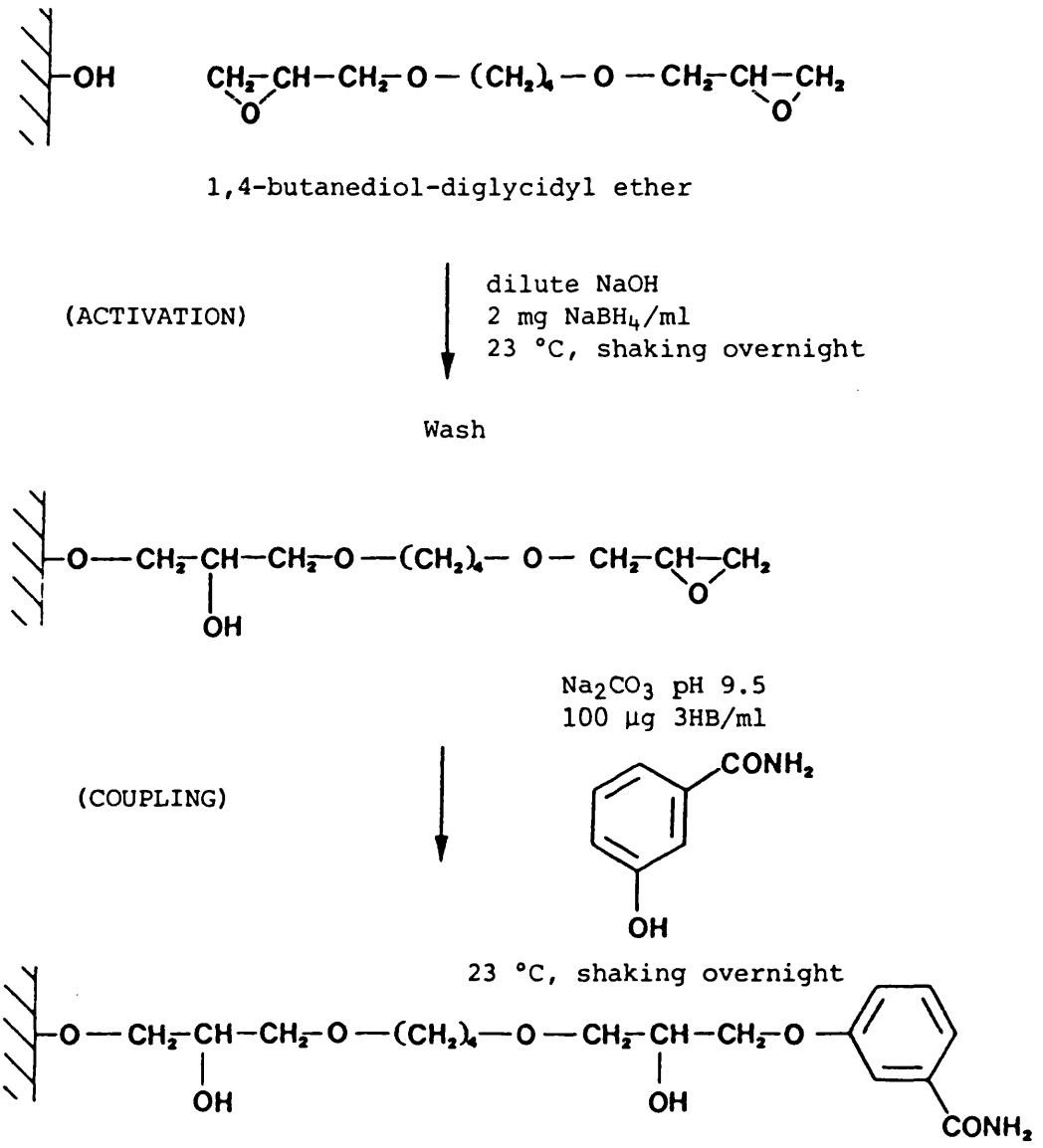


Figure 13 Bisoxirane coupling of 3HB to Sepharose-6B

>40 °C at pH 11-12. The direct result of using such conditions could be reduced or negligible coupling. If it is assumed that coupling efficiency was only 1%, the theoretical capacity of the column would only be 5.2 nmoles of native enzyme, equivalent to 605 µg.

Unfortunately, due to the small quantities of compound used quantitation of coupled ligand could not be determined accurately. Radiolabelled ligands may be used to overcome such problems (section 4.3.8), although labelled 3HB was not available in this case.

Assuming that coupling had been achieved successfully, reduced binding may have resulted from steric hindrance. The degree of displacement of immobilised ligand, as well as the nature of the spacer arm, can be critical (Cuatrecasas, 1970; Jost, 1974). A 6 atom spacer arm had been tried unsuccessfully in this laboratory previously, to purify ADPRT and it is possible that the bisoxirane used in this case was too short to enable efficient interaction.

Non-specific binding to the ligand, due to the hydrophobic nature of the inhibitor (section 4.3) could mask specific binding sites, lowering the apparent column capacity. Another possibility is that the effectiveness of the inhibitor may have been altered by formation of the ether linkage with the spacer. This seems unlikely, as 3MeB remains a potent inhibitor, despite the ether linkage to the methyl group. The bulk, and not the nature, of the substituted group seems more important to the potency of the molecule as an inhibitor for steric reasons alone.

Although oxirane coupling does have advantages over other methods, in that the linkage is stable and free of charge, the experimental observations indicated that Sepharose-6B derivatised in this way was

not worthy of further pursuit. Coupling efficiency could possibly be improved by increasing the concentration of inhibitor in the coupling mixture, prolonging the reaction time or increasing the temperature. Unfortunately, the cost and availability of 3HB did not allow these possibilities to be explored.

Use of divinyl sulphone

Divinyl sulphone may be more suitable for linking 3HB to agarose, due to the greater reactivity of the vinyl groups (relative to oxiranes) and hence the lower pH and temperature required for coupling (Porath, 1972). The linkage is unstable above pH 9 to pH 10, but would only prove limiting if excessive pH was necessary for binding or elution. The length of the spacer may be inhibitory sterically and excessive cross-linking can occur, although the resulting improved flow qualities may be a compensatory factor.

4.3.3 Affinity chromatography using 3AB

Coincident with the attempts to synthesise a hydroxybenzamide based affinity matrix, a method was published for affinity purification of human placental ADPRT using the succinylated derivative of 3AB as an affinity ligand (Burtscher *et al.*, 1986).

The matrix was composed of cyanogen bromide activated Sepharose-4B coupled to a linear linker, bisiminopropylamine, followed by carbodiimide mediated attachment of the inhibitor. The protocol presented an apparent 4900 fold purification.

The effectiveness of the method, augmented by the requirement of purified enzyme at the earliest possible opportunity, led subsequently to concentration of effort towards adoption of this affinity method.

4.3.4 Cyanogen bromide activation

Mild coupling of aromatic and aliphatic amine containing species to insoluble polysaccharide supports is routinely carried out *via* cyanogen bromide activation of the support (Axen, 1967; Porath, 1967, 1968; Cuatrecasas, 1968). Highly reactive cyanate esters are formed which will readily couple to unprotonated amines. These groups do undergo hydrolysis to inactive carbamate, as well as rearrangement to slightly active linear and cyclic imidocarbonates, but the ester is more prevalent (∿80%) (Wilchek *et al.*, 1984).

Coupling of ligand results in N-substituted carbamates, N-substituted imidocarbonates and N-substituted isoureas with the latter predominating (Axen, 1971; Svensson, 1973; Hofstee, 1973; Wilchek *et al.*, 1975).

The use of cyanogen bromide to activate polysaccharide supports is hampered by a number of inherent problems.

As well as the highly toxic nature of the reagent itself, the activation reaction gives rise to two species of ionisable groups in the coupled product. The N-substituted imidocarbonates are weakly basic and therefore do not contribute at physiological pH. The N-substituted isoureas, however, have pK values of about 10 and under such conditions confer a positive charge onto the column. The column thus exhibits ion-exchange properties (Axen and Ernback, 1971; Svensson, 1973; Jost *et al.*, 1974; Wilchek *et al.*, 1975).

Detergent like behaviour has been recognised in some cases as a result of this charge contribution and the hydrophobic nature of some spacer molecules. Coupling of dihydrazines to cyanogen bromide activated agarose has been described, therefore, as a means of

eradicating such non-specific ionic and hydrophobic interactions (Jost *et al.*, 1974).

Leakage of attached ligand from cyanogen bromide activated supports can also be a problem, due to the susceptibility of the isourea group to nucleophilic attack (Tesser *et al.*, 1972; Wilchek *et al.*, 1975). Buffers containing amines (except Tris, which has its amine group sterically shielded) should be avoided, as they can lead to release of N_1-N_2-disubstituted guanidines from the matrix (Wilcheck *et al.*, 1975). This tends to be a more serious problem when the ligand attachment is monovalent, rather than multivalent.

4.3.5 Matrix synthesis (Figure 14)

Activation and coupling of spacer to Sepharose-4B

The activation step was carried out largely by the method of Cuatrecasas (1970).

50 ml well washed Sepharose-4B was mixed well with 50 ml distilled H_2O and 10 g finely divided cyanogen bromide, using a magnetic stirring bar. The temperature was maintained at 20 °C by addition of ice and the pH maintained at pH 11 by titration with 4 M NaOH. After cessation of proton release (approximately 10 minutes), two volumes of crushed ice were added to the suspension which was rapidly vacuum filtered through a coarse disc on a Büchner funnel. This was followed by washing with 1.5 litres ice-cold sodium carbonate buffer pH 9.6. 20 mmoles bisiminopropylamine (Aldrich) in 50 ml carbonate buffer were then added to the gel, followed by gentle stirring with a glass rod. Due to the high reactivity of the cyanate esters, this step was carried out as quickly as possible (<2 minutes). The suspension was then transferred to a beaker which was stirred gently at

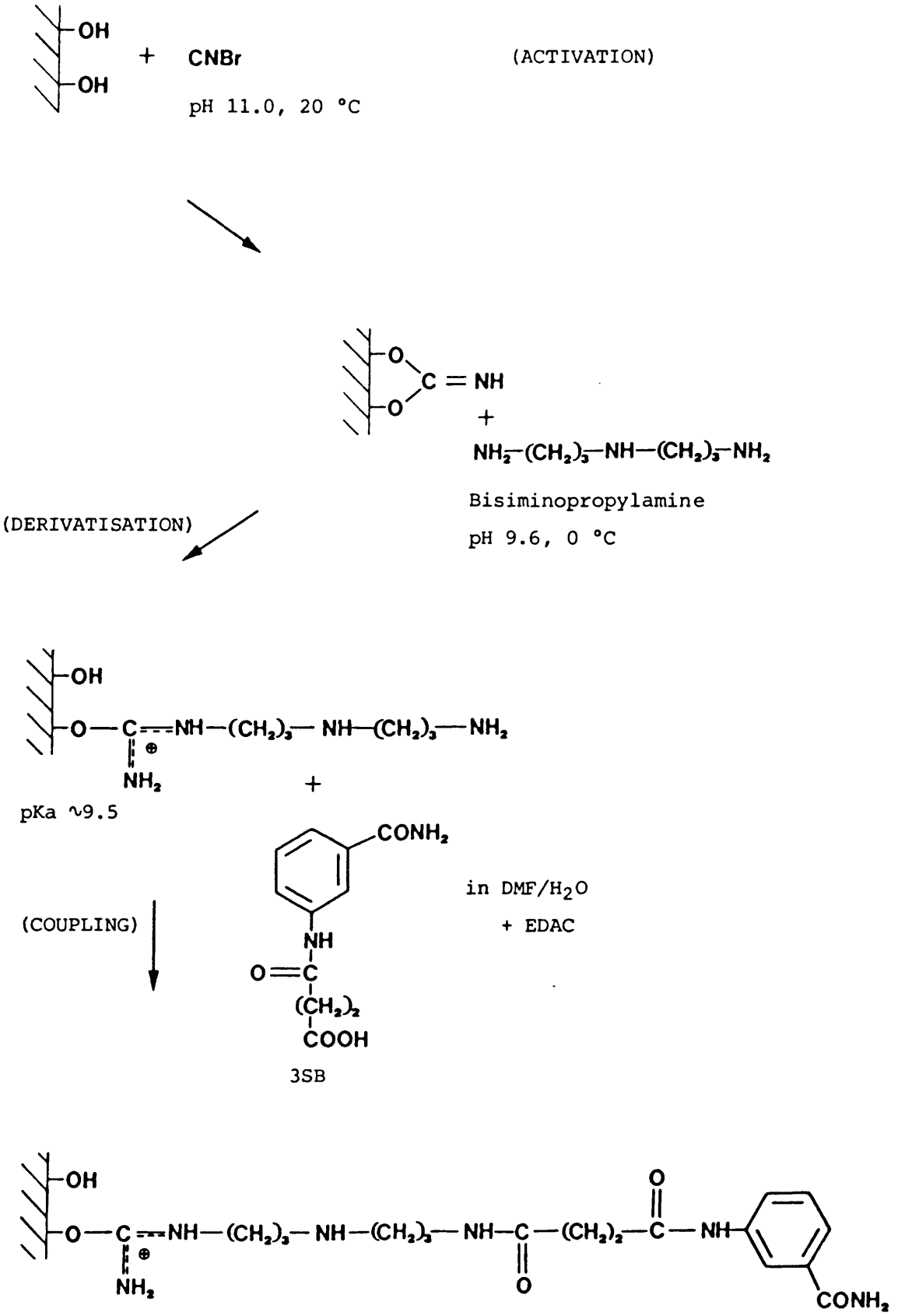


Figure 14 Coupling of 3SB to Sepharose 4B *via* cyanogen bromide activation.

4 °C for 18 h to ensure loss of reactive groups on the agarose. The coupled gel was then suction filtered, followed by extensive washing with distilled H_2O to remove unbound ligand.

Attachment of inhibitor

0.5 g of the succinylated inhibitor (Purnell, 1980) (2.1 mmoles) was dissolved in 20 ml dimethylformamide (DMF) and added to 25 g (wet weight) derivatised Sepharose. 0.75 g 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide (EDAC-Sigma), dissolved in 3 ml H_2O, was added and the mixture shaken gently overnight at 20 °C. The affinity matrix was washed with 200 ml 50% v/v DMF and then extensively with distilled H_2O. The prepared gel was again stored in 15% v/v ethanol, to protect the amide group on the ligand.

4.3.6 Preliminary studies

A 1 ml column was poured and equilibrated with SPB. 10 ml HA purified ADPRT was applied to the column as previously and the column washed with 10 ml equilibration buffer, followed by 10 ml eluting buffer (1 mM 3MeB in SPB). The eluate was applied directly to a 1 ml HA column, followed by a further 10 ml wash of the column to remove inhibitor. Bound enzyme was recovered with 5 ml 0.6 M potassium phosphate buffer pH 8.0.

		Enzyme activity (dpm x 10^6)	Recovery (%)	Protein (µg)
	HA purified material	22.4 ± 0.28	100	36 670
	Post affinity material	1.1 ± 0.1	4.7	26 300
	Affinity wash	-	-	1 530
	3MeB eluate	-	-	85
Inhibitor separation	Post HA	-	-	15
	HA wash	-	-	0
	HA eluate	0.16± 0.01	0.7	85

The column apparently retained 95% of the enzyme activity applied, which is indicative of successful coupling and available ligand. Although this was encouraging, a number of areas needed clarification, however, not least of which was the very poor recovery of enzyme activity observed.

Less than 1% of the enzyme activity apparently retained by the affinity column was observed in the final eluate. This was probably due to one or more of the following possibilities:

(1) Gel substitution was not carried out with strict regard for low degrees of coupling. If the matrix had been supersaturated with ligand, it was possible that interaction was too tight to facilitate elution under the conditions described.

(2) Unsuccessful separation of enzyme from inhibitor by the 1 ml HA column. More stringent washing would therefore be necessary, in order to observe activity.

(3) The assay itself could have been responsible for giving misleadingly low levels of enzyme activity. Histones were not present in the assay at this stage and if ADPRT was the only protein present, then the very low levels of acid insoluble product may have been due primarily to automodification of the enzyme alone.

(4) A more worrying possibility was that the inhibitor was being released from the support due to nucleophilic attack by one or more components of the enzyme extract, at the isourea site.

The presence of leached ligand in the enzyme extract after passage through the column may have been responsible for the observed low levels of enzyme activity in the affinity column effluent.

The high level of protein bound to the column was disappointing, but not unexpected. 24% of the applied material bound to the supposedly biospecific adsorbant. For reasons already discussed, however, the adsorbant could exhibit pronounced hydrophobic properties. This factor, coupled with electrostatic retention of proteins by charged isourea groups, could explain the apparently high degree of non-specific binding observed.

4.3.7 Assessment of coupling and leaching from cyanogen bromide activated Sepharose-4B

As stated in section 4.3.2, the assessment of substituted efficiency and leaching of ligand from cyanogen bromide activated Sepharose-4B is difficult in this case, due to the nature of the substitution required. These problems may be overcome, however, by inclusion of some radioactive ligand in the coupling reaction leading to attachment of measurable quantities of the radioactive component to the insoluble support. Knowing the specific activity of the ligand in the mixture, the efficiency of coupling, and the subsequent release of coupled ligand, may be determined accurately.

4.3.8 Synthesis of radioactive 3-succinimidylbenzamide (3SB) (Purnell 1980)

To 50 µCi [1,4-^{14}C] succinic anhydride (120 mCi/mmol Amersham) was added 400 µl dry pyridine. An approximate 10 fold excess of 3AB (0.57 mg) dissolved in 100 µl pyridine was added and the reaction left overnight at room temperature. 0.5 ml distilled H_2O was then added and after one further hour at room temperature, the solvent was evaporated from the product using N_2 gas. The product was analysed by TLC (section 2.5).

Product analysis

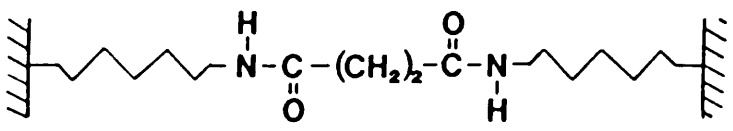
A small amount of the product mixture was spotted onto the origin of a TLC plate, alongside cold 3AB and 3SB standards, using an extruded melting point tube, and the spots dried in a warm air stream. The plate was then placed in a glass developing tank, containing 0.4 cm of the butanol solvent system in the bottom, the lid was replaced and the plate left until the solvent front had migrated 10 cm from the origin. The plate was then dried in a stream of warm air.

Counting of radioactivity

The chromatogram was cut into 1 cm strips which were placed in scintillation vials containing 1 ml 0.5 M perchloric acid. The vials were incubated for 30 minutes at 100 °C, after which 10 ml Optiphase was added, followed by mixing. After cooling, the vials were counted for radioactivity as previously (Figure 15).

From the profile, it could be seen that 44% of the radioactivity present did not migrate from the origin, corresponding to succinic acid. 46% of the radioactivity migrated with an Rf value similar to that observed for the succinylated standard and was assumed to be the radioactive product.

The presence of so much succinic acid probably resulted from trace amounts of water in the pyridine used. The presence of the succinic acid produced can lead to intra- and inter-bead cross-linking when coupling to the derivatised Sepharose is attempted: *i.e.*,



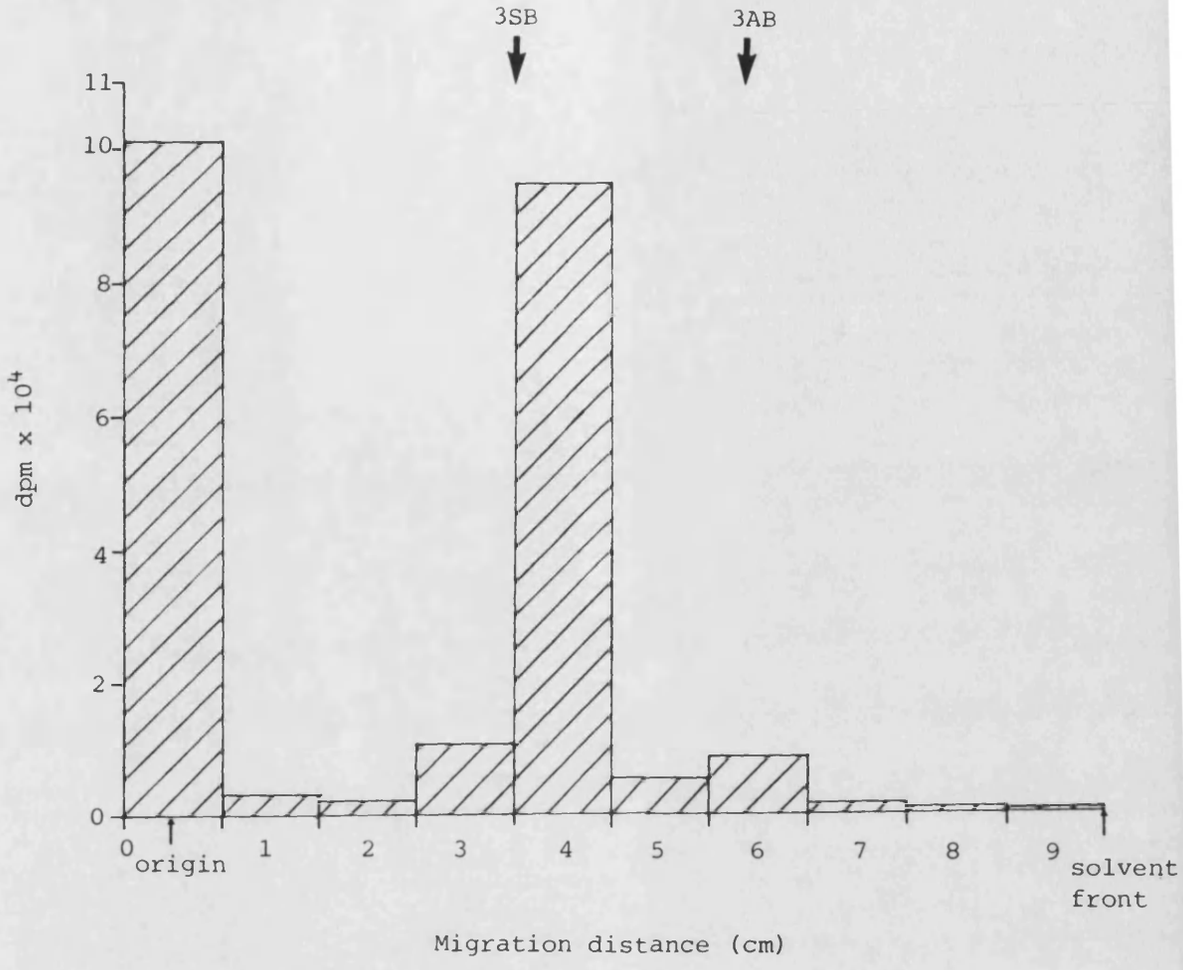


Figure 15 TLC profile of reaction product developed in the butanol/H_2O/methanol/NH_3 system as described in section 2.5.

In carrying out leaching studies, this would introduce a complication, in that release of radiolabel will only occur as a result of nucleophilic attack at both sites of attachment. The remaining succinic acid, and the excess 3AB, which would compete with the primary amine on the spacer arm in the coupling reaction, therefore had to be removed from the succinylated inhibitor.

Preparative TLC

Due to the very small amount of succinylated product, an analytical PEI-impregnated cellulose plate was chosen for the separation.

The origin of the plate was divided into an 'analytical' and a 'preparative' section. To the analytical section, 0.75 cm from the left hand edge of the plate, was applied less than 1 µl of the mixture. A further 0.75 cm gap was left to the right of the spot, before the remaining mixture was applied evenly along the preparative origin. The chromatogram was run in the butanol system until the solvent front had migrated approximately 10.5 cm from the origin. After drying, the two sections of the plate were separated and the analytical portion counted for radioactivity (Figure 16).

Although separation was achieved successfully, as in the previous experiment, one cause for concern was the apparent increase in the ratio of radioactivity of succinic acid to inhibitor. Where the ratio was previously 1:1.045, the TLC showed an increase to a ratio of 1:0.78, indicating possible hydrolysis of the 'peptide-like' linkage (assuming experimental error was not responsible).

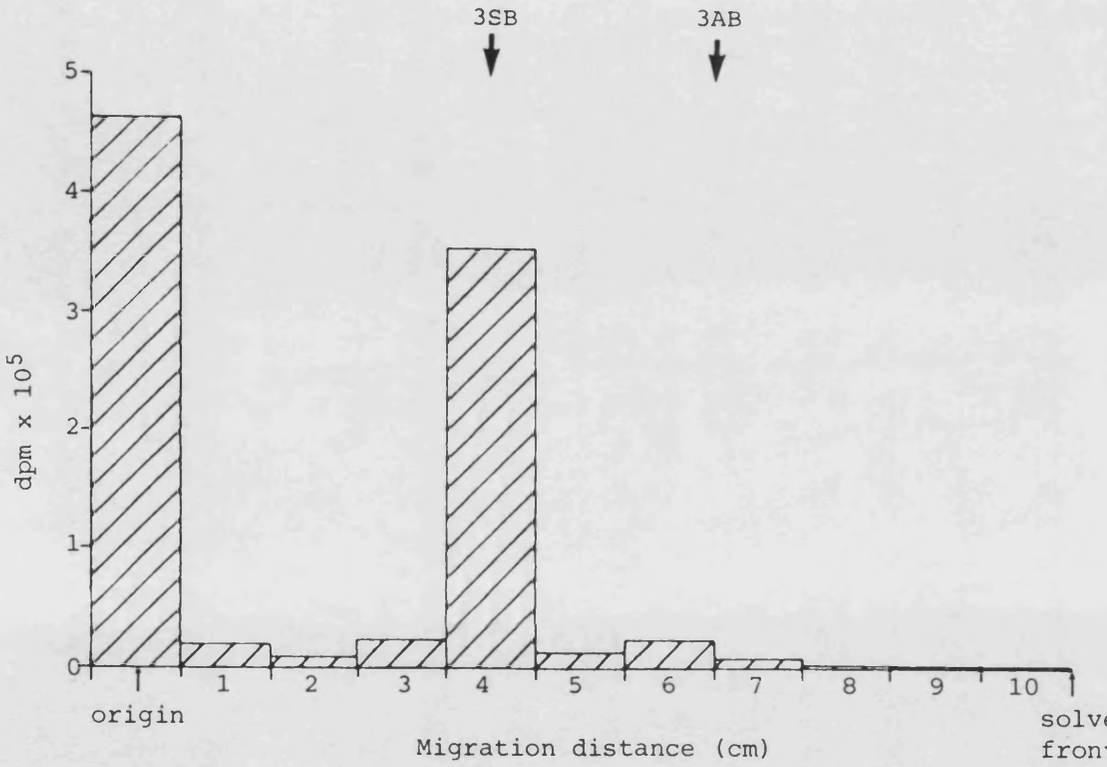


Figure 16 Analytical TLC profile of preparative plate developed in the butanol/H_2O/methanol/NH_3 system (section 2.5).

Recovery of derivative from the plate

The PEI-cellulose containing the product was scraped from the plate (in a fume hood) into a clean glass tube (2.5-6 cm from the origin). The powder was then washed successively with 3 ml volumes of the developing solvent system, followed by centrifugation, until negligible radioactivity was released from the support. The washes were pooled in a 50 ml round bottomed flask and the solvent removed by rotary evaporation. The resulting solid was dissolved in 2 ml DMF, followed by TLC analysis (Figure 17). 5 µl of the solution was also counted for radioactivity in 3 ml optiphase.

The total amount of radioactivity recovered was 19.2 µCi. Surprisingly, however, only 67% migrated in the manner expected, with a significant amount of activity remaining bound to the origin.

The proposed hydrolysis of the carboxamide linkage seemed to be borne out by this observation. The presence of ammonia in the solvent system, particularly during the evaporation step where the solvent was heated, was probably the cause. Different separation and recovery solvent systems therefore had to be utilised in order to recover homogenous labelled ligand.

Evaluation of different TLC solvent systems

Separation of the mixture was attempted on PEI-cellulose plates using the two different solvent systems, 100 mM acetic acid and 200 mM $LiCl_2$. The plates were loaded as previously and run until the solvent front was 14.5 cm from the origin. The plates were run with standards in parallel and counted for radioactivity (Figure 18).

Both systems were equally suitable for separation of the constituents of the mixture. However, $LiCl_2$ was preferred over acetic

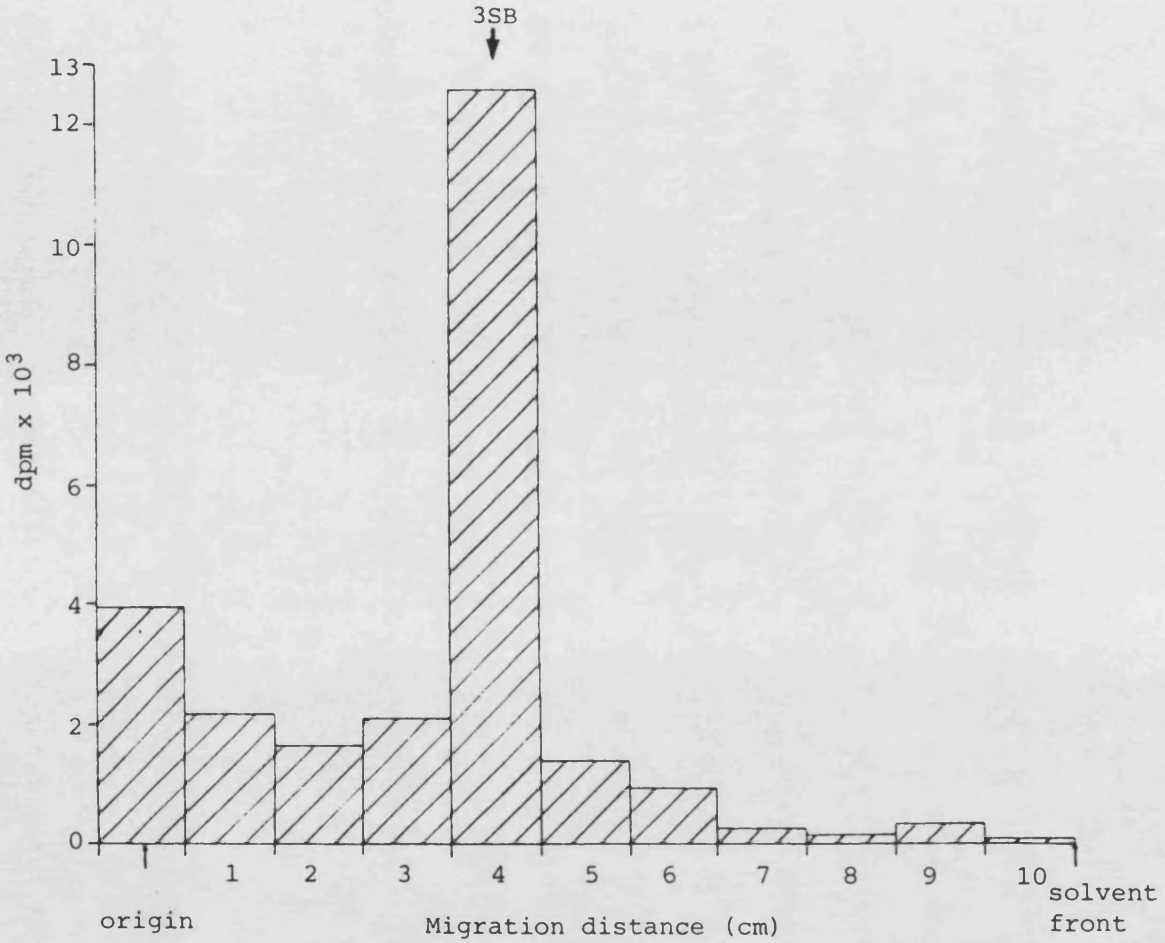


Figure 17 TLC profile of recovered product developed in the butanol/H_2O/methanol/NH_3 solvent system (section 2.5).

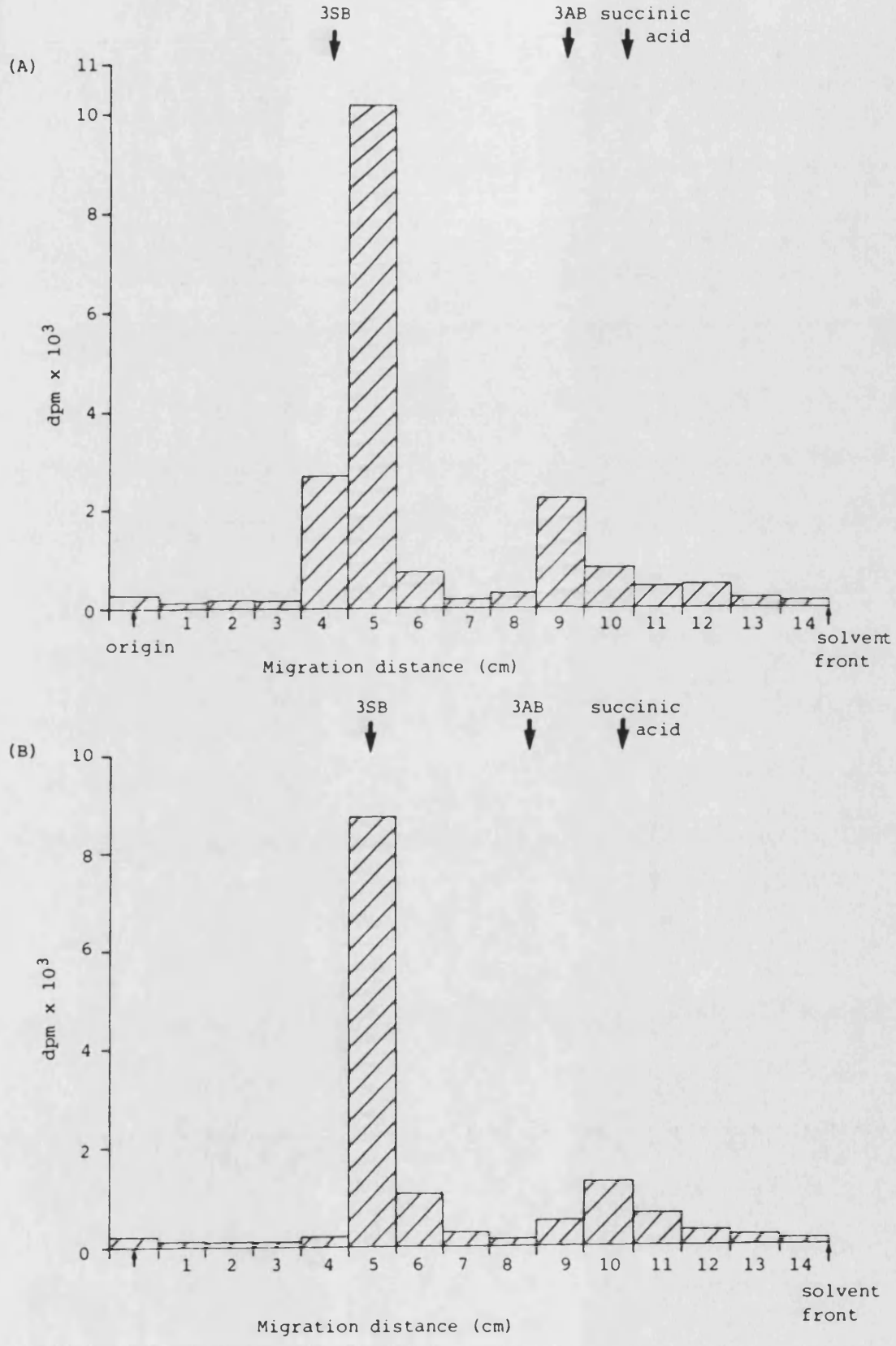


Figure 18 TLC profiles of recovered product developed in 100 mM acetic acid (A) and 200 mM $LiCl_2$ (B)

acid, due to the relative harshness of the latter.

Recovery of bound product

As elution with the butanol system could not be applied, due to the instability of the product, several other solvents were tested for elution.

Eleven 5 µl aliquots of radioactive sample, diluted 1 in 6, were spotted onto strips of PEI-cellulose. When dry, the plate strips were cut up and placed in scintillation vials. 3 ml of each of the different solvent systems were added to individual vials, the vials capped, and the contents mixed by vortexing. The vials were left for 30 minutes at room temperature, vortexed again and 1 ml aliquots transferred to microfuge tubes. The tubes were spun for 2 minutes at high speed in a microfuge and two 400 µl aliquots counted for radioactivity in 5 ml Optiphase. Two 5 µl aliquots of the original mixture were also counted (Figure 19). Three solvent systems showed 100% elution. 90% DMF in water was chosen for further elution, however, due to its mild nature and ease of removal by evaporation.

Preparative TLC in 200 mM $LiCl_2$

A PEI-cellulose plate was loaded as previously, in two sections, and run for 13 cm in 200 mM $LiCl_2$. The analytical section was cut into 0.5 cm sections and processed as previously, with perchloric acid, prior to scintillation counting (Figure 20). The corresponding preparative section of the plate containing inhibitor, between 3.5 and 7.5 cm was scraped into a clean glass tube and 50 ml 90% v/v DMF added. The tube was vortexed and spun in a bench centrifuge at 4000 rpm for 2 minutes. The supernatant was transferred to a 50 ml round bottomed flask and the wash procedure repeated until no

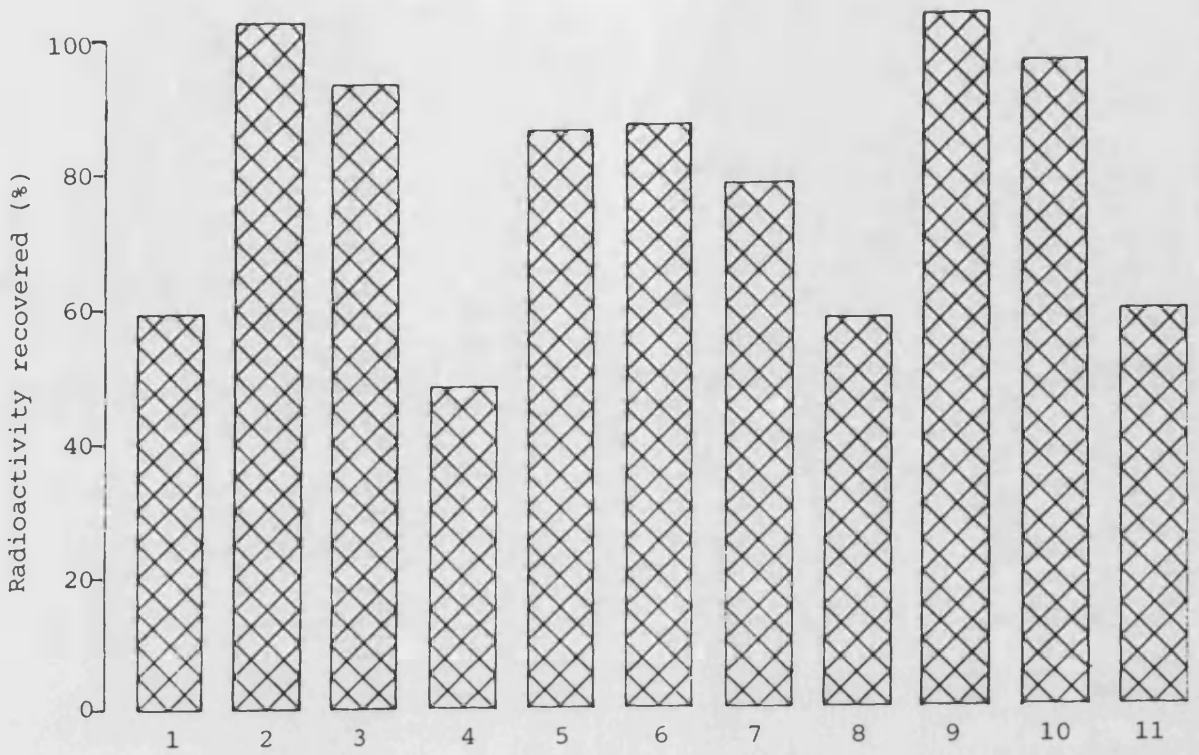


Figure 19 Elution of radioactive 3SB from PEI-cellulose.

The 11 solvent systems listed below were evaluated for their ability to elute the radioactive inhibitor from PEI-cellulose.

1 DMF
2 90% v/v DMF
3 50% v/v DMF
4 90% v/v ethanol
5 50% v/v ethanol
6 20% v/v methanol, 60% v/v butanol, 20% v/v H_2O
7 90% v/v methanol
8 50% v/v methanol
9 200 mM $LiCl_2$
10 100 mM acetic acid
11 H_2O

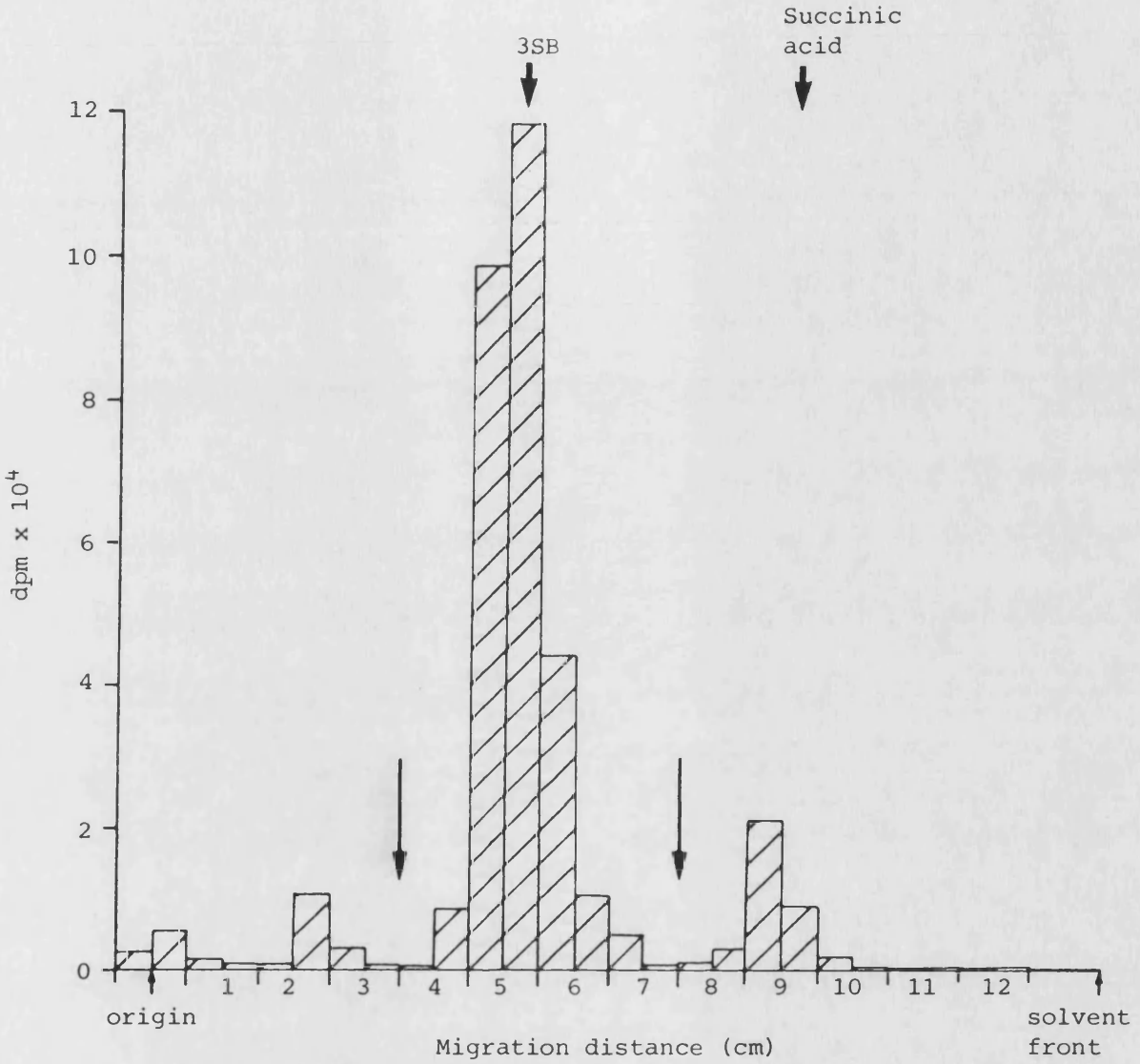


Figure 20 Analytical TLC profile of 'Preparative' plate developed in 200 mM $LiCl_2$.

The area between the two large arrows was scraped and the material processed as described in the text.

significant activity was released from the support (<5000 dpm/ml). The pooled volume (approximately 30 ml) was reduced to approximately 2 ml by rotary evaporation and transferred to a 10 ml pear-shaped flask. The large flask was repeatedly rinsed with DMF, followed by transfer to the small flask and reduction, until the final volume in the small flask was less than 1 ml. The contents were finally transferred to a 1.5 ml microfuge tube, plus a 0.5 ml DMF wash. The tube was vortexed and two 5 µl aliquots counted for radioactivity in 2 ml Optiphase.

The total volume was 840 µl (by weight) with a specific activity of 14.5 µCi/ml.

Final product analyses

Two final TLC plates were run to determine the purity of the product using the $LiCl_2$ and butanol systems (Figure 21).

Only one peak was seen in the $LiCl_2$ system with an Rf value corresponding to the succinylated inhibitor. The butanol system, however, showed two peaks with only 41% present as inhibitor.

It is possible, therefore, that while it was assumed that the original reaction had been partially successful, with only 46% of radioactivity migrating with the Rf expected of the product, the amount of succinyl derivative formed may have been much greater, *i.e.*, product hydrolysis appears to have occurred with the butanol system. If the $LiCl_2$ system had been used initially, the overall recovery of radioactivity may have been much higher than that actually observed (24%).

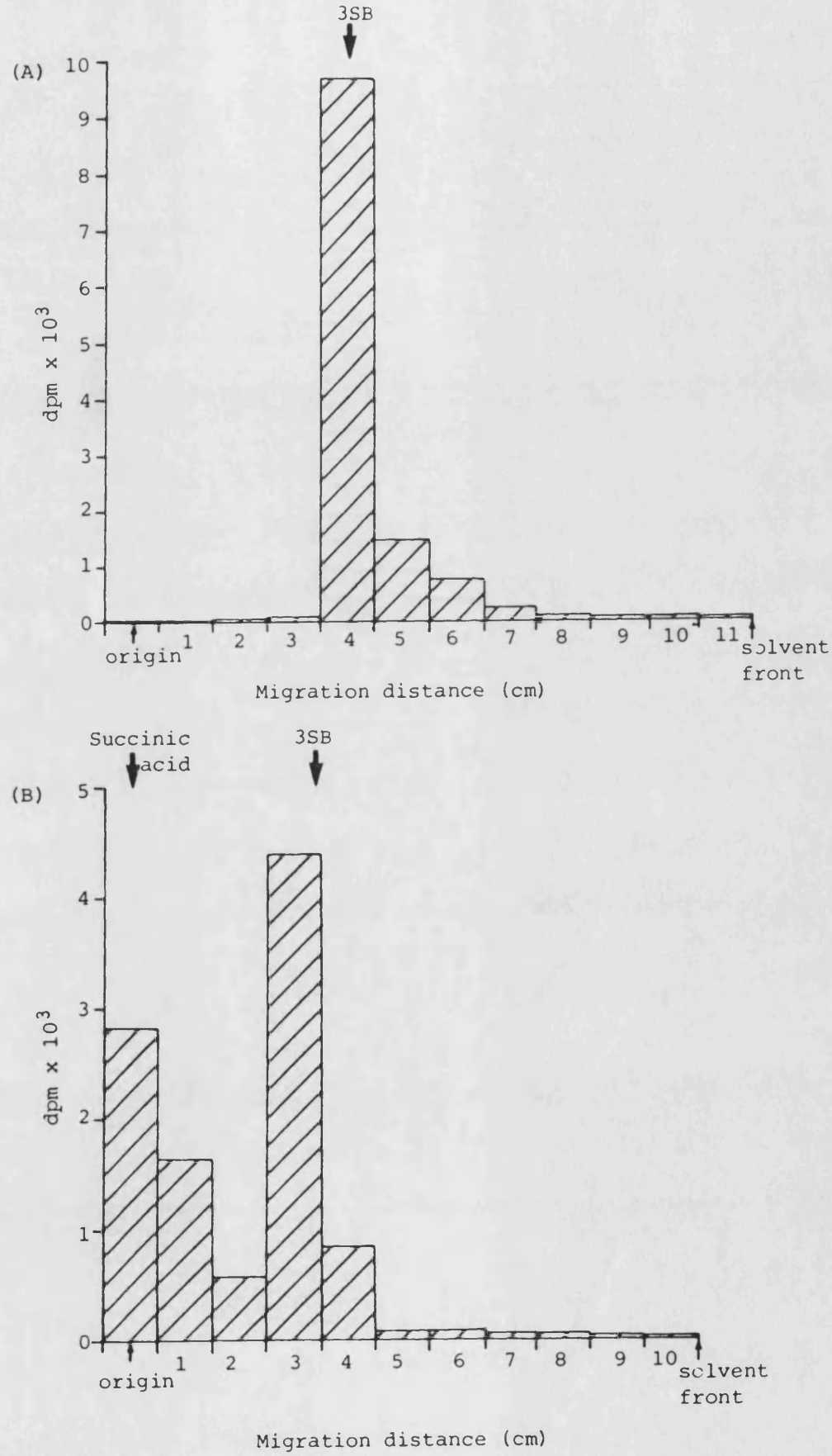


Figure 21 TLC profiles of final purified product developed in 200 mM $LiCl_2$ (A) and the butanol/H_2O/methanol/NH_3 system (B).

4.3.9 Coupling of radioactive inhibitor to derivatised Sepharose

5 µCi of radioactive ligand in 3 ml DMF were added to 5 ml packed volume of derivatised Sepharose. 200 mg EDAC in 1 ml H_2O was added and the mixture swirled at 20 °C for 16 h. The coupled gel was then suction rinsed with 300 ml 50% v/v DMF on a Büchner funnel (GF/C disc) followed by 300 ml H_2O to remove unbound inhibitor. The gel was transferred to a conical, graduated glass tube and the H_2O replaced with 80% v/v ethanol, to a total of 10 ml. Two 100 µl aliquots of supernatant and three 50 µl aliquots of vortexed slurry were counted for radioactivity in 3 ml optiphase.

Quenching of disintegrations by the agarose beads was not a problem, due to the relatively high energy of ^{14}C emissions.

Coupling efficiency

Radioactivity of bead suspension per 50 µl = 4750 dpm.

Total radioactivity in coupled matrix = $4750 \times \frac{10000}{50}$ dpm

$= 9.5 \times 10^5$ dpm

$= \underline{0.43\ \mu Ci}$

Percentage coupled $= \frac{0.43}{5.0} \times 100$

$= \underline{8.6\%}$

43.10 Leaching of coupled ligand

The coupled gel was stored in the dark at 4 °C and analysed at regular time intervals for release of radioactivity.

At each interval the tube was gently mixed, followed by pelleting of the beads in a bench centrifuge. Three 50 µl aliquots of the supernatant were then counted for radioactivity in 3 ml Optiphase.

After 45 days, the gel was washed extensively and the 80% v/v ethanol replaced by 0.1 M TEA.HCl pH 8.0. The above sampling procedure was repeated and the aliquots counted for radioactivity (Figure 22).

3.2% of coupled ligand was released after 45 days in 80% v/v ethanol. Unexpectedly, however, after 42 days in buffer, only 1.9% of the ligand was released, although this probably reflects increased hydrophobicity in the absence of the organic solvent. Should this be the case, the measurement carried out in buffer is probably an underestimate.

Protein extracts contain a complex mixture of nucleophilic species, and even if it is assumed that release in 80% v/v ethanol approximates to behaviour in the presence of such a mixture, the apparent low recoveries of enzyme activity could not be explained by the presence of leached inhibitor. Even if the column substitution was as high as 40 µmoles per ml, this would account for only 28 nmoles of released inhibitor per day (if leaching occurred in a linear manner).

Unless the HA purified enzyme solution contained one or more powerfully nucleophilic species, the apparently low recovery of enzyme activity recorded thus far must have been due to retention on the column.

4.3.11 Contributions to binding by ionic interactions (effect of salt)

Ionic contributions were assessed with a view to different elution methods.

Eight tubes containing 50 µl packed volume affinity adsorbant were set up on ice. To each tube was added 350 µl SPB, containing varied amounts of NaCl, and 100 µl of HA purified enzyme. The final

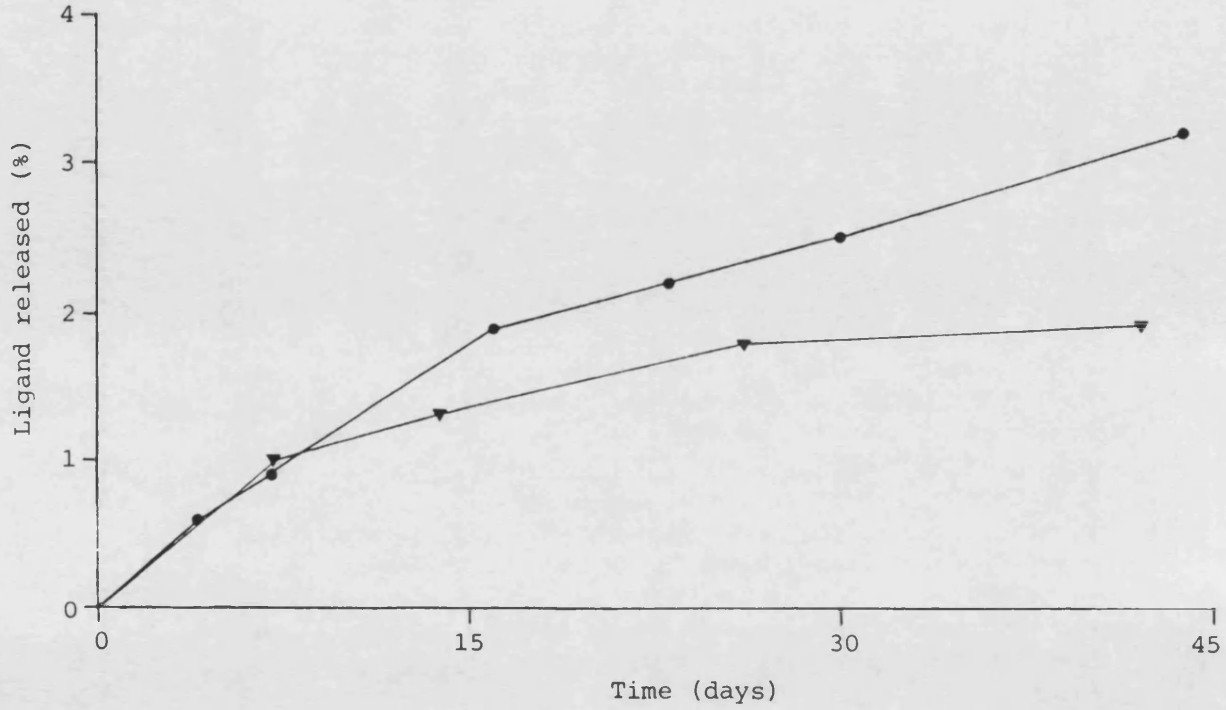


Figure 22 Leaching of ligand from affinity matrix.

Release of ligand was determined in the presence of 80% v/v ethanol (●) and 0.1 M TEA.HCl pH 8.0 (▼).

salt concentrations varied from 0.2 to 3.0 M. Each tube was vortexed immediately, then at approximately 5 minute intervals up to 20 minutes, followed by pelleting of the gel in a bench microfuge. The supernatant of each tube was assayed in duplicate (50 µl) for enzyme activity.

A 100% control was set up containing buffer only instead of gel, without added salt. Unfortunately, the tube contents were lost and the results could therefore only be presented in the form of observed activity against salt concentration (Figure 23).

The results showed definite dependance on low ionic strength for binding. The effect was approximately linear to 1 M salt, followed by a gradual decrease in observed activity above this level. This fall off in activity did not signify changes in binding characteristics at high ionic strength, but did reflect salt inhibition of enzyme activity in the assay.

If binding is ionic strength dependant, it follows that elution may also be approached from this viewpoint and non-specific elution of the adsorbant was thus attempted.

Salt elution of affinity column

A 1 ml affinity column was poured and washed with SPB. 50 ml HA purified material was applied to the column, followed by a 20 ml buffer wash. 4 ml eluting buffer (2.5 M NaCl in SPB) was then passed through the column twice and the eluted material assayed for enzyme activity and protein.

In order to determine any deleterious effects caused by high salt concentrations, the following experiment was also carried out.

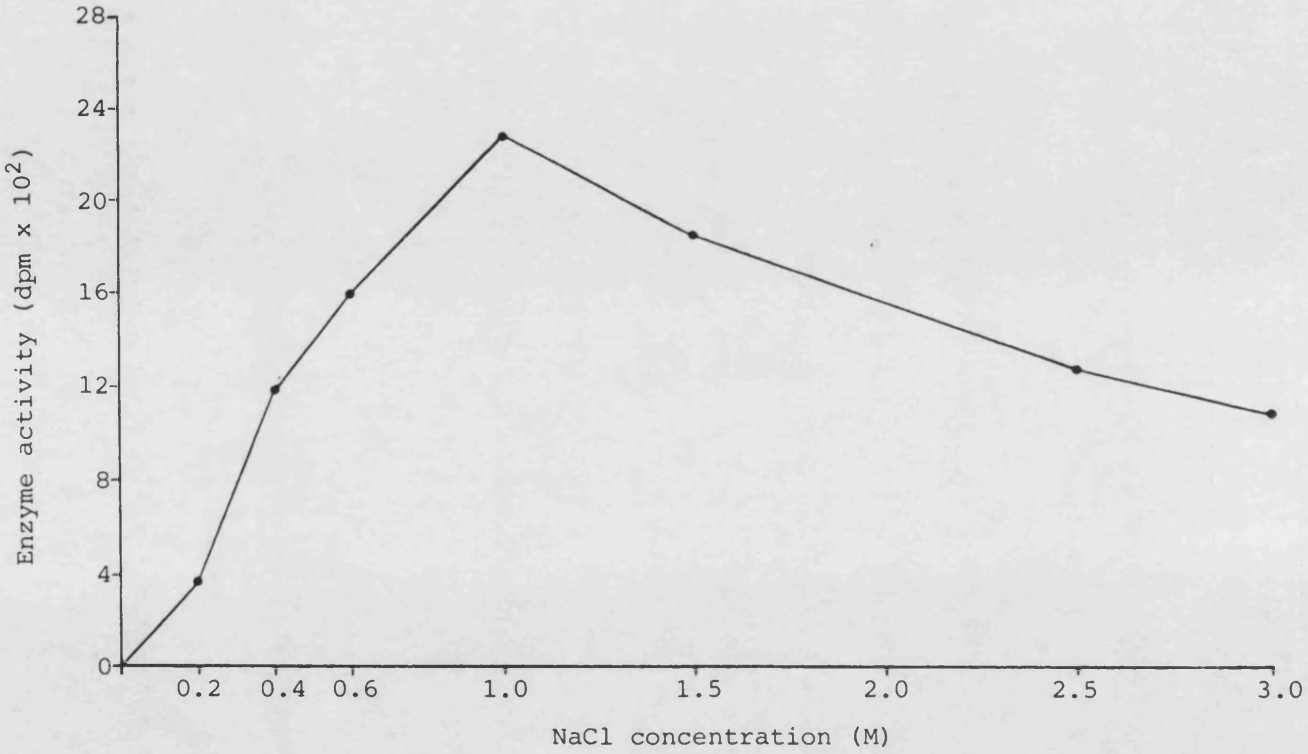


Figure 23 Effect of salt on enzyme/matrix interactions.

Binding of enzyme to the affinity matrix was quantified over a concentration range of 0.2-3.0 M NaCl (section 4.3.11).

Two microfuge tubes, each containing 100 µl HA purified material, were set up and placed on ice. To tube A was added 300 µl 4 M NaCl in SPB and to tube B 300 µl SPB only. The tubes were vortex mixed and left on ice for 30 minutes before assaying in triplicate, under standard conditions for enzyme activity.

Sample	Enzyme activity (dpm x 10^8)	Protein (mg)
HA purified material	1.01	10.5
Post affinity material	0.11	9.25
Affinity wash	0.01	0.52
Salt eluate	0.06	0.276

11% of the activity applied to the column was recovered in the column effluent and wash, and only 6.7% of the retained activity was observed in the salt eluate. 38% of retained protein was recovered, leaving approximately 0.5 mg bound by means other than of a charge nature.

Sample	Enzyme activity (dpm x 10^4)
HA purified material + 3 M NaCl	5.34 ± 0.69
HA purified material + buffer	5.64 ± 0.32

Irreversible salt effects were not to blame for low recovery, even though the assay salt concentration was 120 mM. No significant difference in activity was observed in the presence and absence of salt.

4.3.12 Further studies on ionic interactions (effect of lowered pH)

As the previous experiment had shown ionic strength to be a factor involved in binding of enzyme to the ligand, the effect of pH drop was assessed.

Eight tubes were set up, tubes 1 to 4 containing 0.5 ml SPB and tubes 5 to 8 containing 0.5 ml packed volume affinity adsorbant. 2 ml HA purified enzyme in SPB pH 8.0 was added to each tube and binding allowed to take place for 1 h at 4 °C, during which the tubes were mixed regularly by gentle inversion. The gel in tubes 5 to 8 was then pelleted in a bench centrifuge and washed once in 2 ml SPB. 2 ml volumes were then removed and the following additions made to the tubes.

Tube Number	Addition (1.5 ml)
1 and 5	0.1 M potassium phosphate pH 8.0
2 and 6	0.1 M potassium phosphate pH 8.0 + 4 M NaCl
3 and 7	0.1 M potassium phosphate pH 6.0
4 and 8	0.1 M potassium phosphate pH 6.0 + 4 M NaCl

The tubes were shaken gently for 2 h at 4 °C, followed by centrifugation to pellet the gel. Aliquots were then taken from each tube and assayed for enzyme activity. Percentage recovery of enzyme for each of tubes 5 to 8 was calculated and correction made for irreversible inhibition effects from the results of tubes 1 to 4.

Conditions employed	Total recovery (%)	Inhibition (%)	Corrected recovery (%)
pH 8.0	0	0	0
pH 8.0 + 3 M NaCl	19.2	39.4	31.7
pH 6.0	6.9	29.0	9.7
pH 6.0 + 3 M NaCl	14.5	45.4	26.6

The pH drop did not lead to significant desorption of enzyme activity from the adsorbant. Only 7% of bound activity was released, which corrected for pH effects, still only accounted for 9.7% of bound activity.

As the pK values of carboxyl and amine groups in proteins are usually in the order of pH 3 and pH 9 respectively, the great majority of ionisable groups would be charged at both pH 6 and pH 8. Only if a significant localised change in pK at an interacting site occurred would change in charge have resulted, leading to altered elution characteristics.

The primarily non-ionic nature of the interaction was borne out by the effect of increased ionic strength and pH drop. The increased NaCl concentration did not cause significant alterations in elution characteristics, which would have been expected if charge was a predominant factor.

Hydrogen-bonding may contribute, although probably only through the oxygen on the carboxamide of the inhibitor. The secondary amine at the 3-position would form a hydrogen bond, but is apparently not active in enzyme inhibitor interaction, as demonstrated by the inhibitory potency of unsubstituted benzamide (K_i <1.0 μM) (Shall, 1984). As hydrogen bonding was unlikely to be responsible for such tight interaction, hydrophobic forces appeared to be the predominant form of binding (see hydrophobic affinity chromatography later).

The observed elution of enzyme by high salt probably resulted from disruption of enzyme inhibitor interactions at sites where presentation of the ligand was such that optimal binding did not occur for steric reasons. Sacrifice of the activity recovered in this way was considered as a means of removal of charged contaminants. However, high ionic strength can increase hydrophobic interactions, and though not noticeably damaging ADPRT, can have detrimental effects on a number of proteins.

Although non-specific elution was not pursued further, specific competition being favoured, 0.3 M KCl was subsequently included in all buffers from the HA stage (Burtscher *et al.*, 1986). While this concentration was probably insufficient to remove all non-specific interactions, it was considered a reasonable compromise.

4.3.13 Removal of inhibitor from affinity eluate

Separation of eluted enzyme from inhibitor on HA

As salt elution was largely unsuccessful, specific competitive elution was pursued. The first matter for clarification was the ability of the small HA column to separate inhibitor from eluted enzyme.

Optimal wash conditions were determined by the following experiment.

5 ml HA purified enzyme was mixed well with 5 ml 40 mM 3AB in 5 mM potassium phosphate buffer pH 8.0 and loaded onto a 2 ml HA column. The loaded column was then washed with 100 ml of the same buffer and 1 ml fractions collected. Fractions 24 and 25 were pooled, as were fractions 49 and 50, and 99 and 100. The standard assay was then carried out, using appropriate volumes of the collected fractions in place of assay buffer, on enzyme material from the same preparation. In this way the inhibitory capacity of residual 3AB was detected. The effect of any residual inhibitor was also magnified approximately 20 times, as only 20 µl of material was usually assayed. After the wash, the HA column was eluted with 5 ml 0.5 M phosphate buffer pH 8.0 and the recovered material assayed for enzyme activity using 5 mM potassium phosphate buffer in the reaction mixture.

A 100% activity value was obtained using inhibitor free material from the same preparation, assayed as above.

Sample	Enzyme activity (dpm x 10^3)	Recovery (%)
Pre-inhibitor	8.46 ± 1.16	100
25 ml wash	0.9 ± 0.12	5.7
50 ml wash	7.98 ± 2.55	94.3
100 ml wash	8.79 ± 1.31	103.8

Taking into account the large standard error values and the high volume of wash material in the assay, 50 ml was considered a large enough wash volume for a column of the dimensions chosen.

Of 2.1 x 10^6 dpm applied to the column, 2.4 x 10^6 dpm was recovered, which, allowing for error, accounted for all bound activity.

Use of conditioned HA and gel filtration to separate eluted material from inhibitor

In the previous section, HA proved to be very suitable for removal of inhibitor species from a semi-crude protein preparation. Successful specific elution of the affinity column, however, would result in only microgram quantities of protein being loaded onto the HA column. As new HA had been used in each separation attempted, the possibility of activity loss through non-specific physical binding to the support could not be ruled out. To clarify the problem, aliquots of affinity eluted material were separated on new and used HA and on a gel filtration column.

A 10 ml affinity column was loaded with 100 ml HA purified material and eluted as in the preliminary study (section 4.3.6), using 30 ml 10 mM 3AB, instead of 1 mM 3MeB. The eluate was then

divided into 3 equal volumes. Two of the volumes were applied to two 0.5 ml HA columns, one new and one used, and the third volume placed on ice (see next section). Each column was washed with 30 ml SPB, followed by elution with 2.5 ml 0.6 M potassium phosphate buffer. All fractions were assayed in triplicate for enzyme activity.

Sample	Total activity (dpm x 10^6)	Recovery (%)
Affinity bound enzyme	3.75	100
New HA eluate	0.06 ± 0.02	1.6
Old HA eluate	0.08 ± 0.03	2.1

No significant difference was observed using new and old HA.

Use of gel filtration

BioGel P-10 (BioRad) has an exclusion limit of 17 Kd, which allows inclusion of only unbound inhibitor. Inhibitor associated with ADPRT would pass straight through the column and emerge immediately post void volume.

To determine whether enzyme activity was being masked by tight enzyme-inhibitor interaction, radioactive inhibitor was mixed with an aliquot of the third volume of affinity eluate from the previous experiment, prior to gel filtration. As inhibition by the substituted benzamides is a dynamic phenomenon, addition of radioactive inhibitor would result in proportional separation into enzyme bound and free inhibitor pools. Any bound inhibitor would be excluded from the filtration matrix and appear as a radioactive peak immediately post void volume. The free inhibitor would be included and emerge in a second peak later.

To 200 µl of the affinity eluate was added 0.25 µCi radiolabelled succinylated inhibitor (section 4.3.8), following by mixing. The resulting solution was chromatographed on a 2 ml BioGel P-10 column (10 x 0.196 cm) and drop size fractions collected immediately post void volume. Fractions 11, 13, 15, 17 and 19 were assayed immediately for enzyme activity and the remaining fractions counted for radioactivity (Figure 24).

No enzyme activity was recovered in any of the drops collected and only one radioactive peak, corresponding to free inhibitor, was observed. The presence of only one peak may, however, have resulted directly from dilution of hot inhibitor by cold. One means of overcoming such a problem would be to use inhibitor of high specific activity throughout, although this would prove restrictively cost ineffective in this case.

The lack of enzyme activity observed with gel filtration and HA separations supported the previous suggestion that enzyme was remaining bound to the affinity column.

4.3.14 Time dependance of affinity matrix elution

As stated in section 4.3.6, the nature of column substitution may be critical in terms of binding and elution. Highly substituted columns can give rise to difficulty in elution of specifically interacting protein species, especially involving proteins with high affinity for the immobilised ligand. In such cases, elution may occur throughout the column only for readsorption to take place when the released protein encounters other immobilised ligand further down the column. The observed elution is therefore the net result of the total binding and elution that has taken place.

This is true of absolutely any chromatographic column - Explains nothing

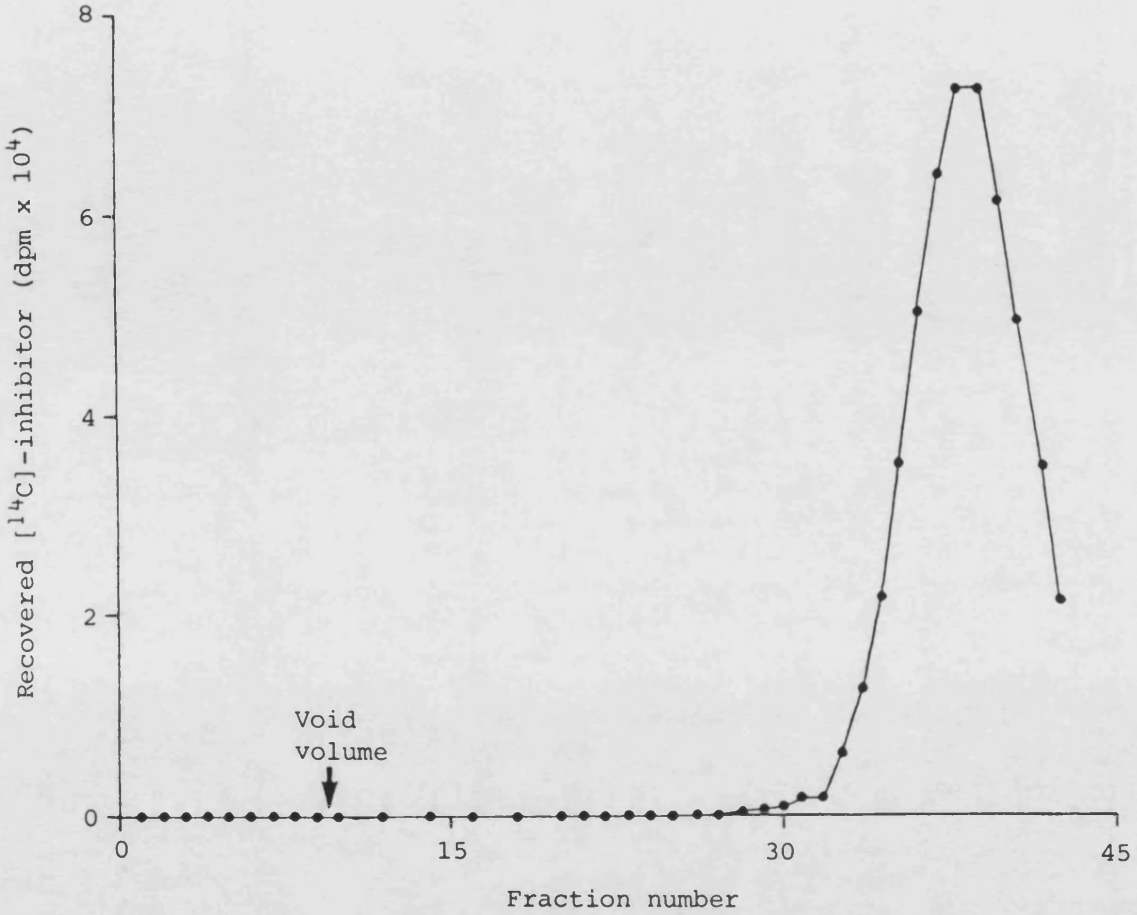


Figure 24 Separation of inhibitor from enzyme by BioGel P-10 chromatography.

200 µl affinity purified material, combined with 0.25 µCi radiolabelled inhibitor and chromatographed on a 2 ml BioGel P-10 column.

Each fraction (1 drop) was counted for radioactivity with the exception of fractions 11, 13, 15, 17 and 19 which were assayed for enzyme activity.

Batch elution of affinity support

Extended exposure of bound species to competing ligand was tried in an attempt to minimise net readsorption of protein.

20 ml HA purified enzyme was applied repeatedly to a 3 ml affinity column, followed by a 30 ml SPB wash. The loaded gel was transferred to a boiling tube clamped over a magnetic stirrer and a stirring bar added. 3AB in SPB was added to a final concentration of 6.7 mM and final volume of 15 ml, and the suspension stirred slowly.

Immediately after addition, and at various time points up to 120 minutes, aliquots of the mixture were taken for assay of enzyme activity in the following way:

2.5 ml of the suspension was transferred to a 10 ml tube and the adsorbant pelleted in a bench centrifuge. The supernatant was applied to the top of a 0.5 ml HA column, followed by a 25 ml buffer wash. Bound material was eluted with 0.6 M potassium phosphate buffer pH 8.0.

No significant elution of enzyme material was observed, even after 120 minutes, indicating that the level of inhibitor was probably too low. The experiment was therefore repeated with a final inhibitor concentration of 20 mM 3AB (Figure 25).

40% of activity was recovered almost immediately, with desorption continuing over the two hour period to a maximum of 72%.

This very encouraging result indicated that specific elution of bound ADPRT was time dependant and that good yields were attainable.

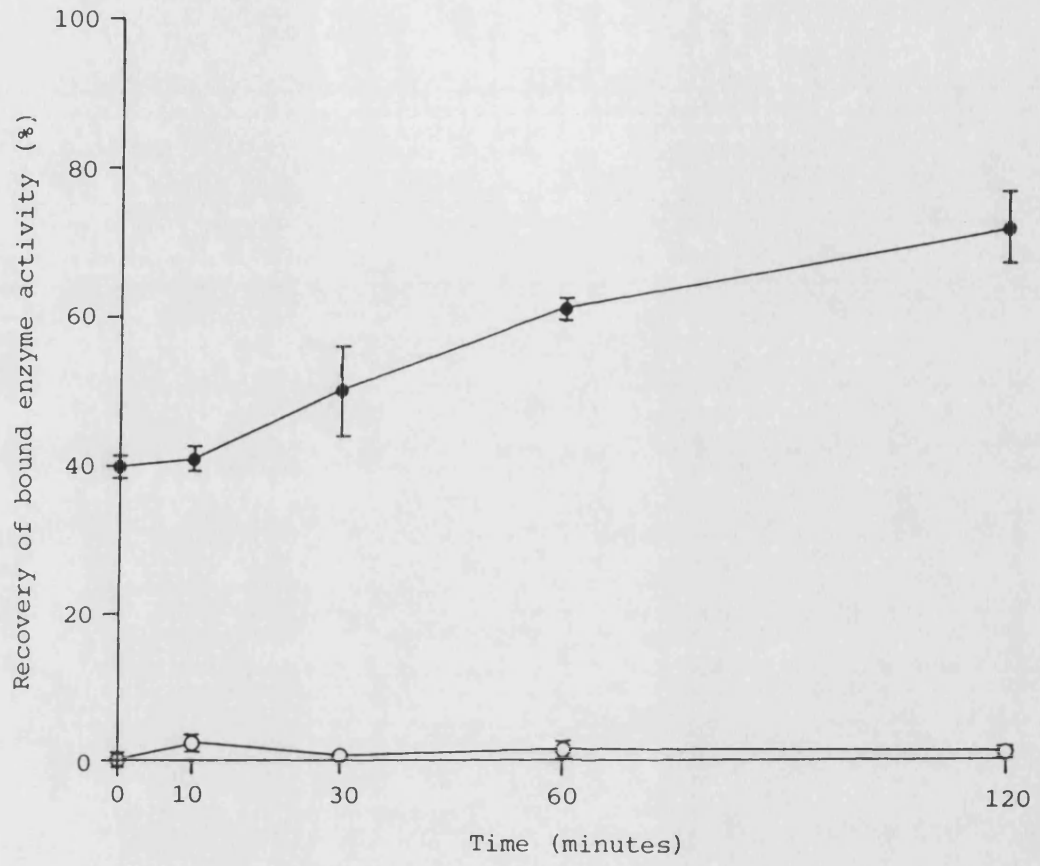


Figure 25 Time dependance of elution from affinity matrix.

Specific batch elution of the loaded matrix was attempted over a period of 2 hours using 6.7 mM (O) and 20 mM (●) 3AB.

Each point represents the mean of 4 determinations and the standard error of that mean.

Nicotinamide elution of affinity column

Application of the previous system on a large scale would require large quantities of inhibitor for successful elution. As this would have been costly in the long run, the effectiveness of nicotinamide as an eluting species was tested.

A column was loaded and eluted exactly as in the preceding section, with the exception that nicotinamide was added to the loaded adsorbant, to a final concentration of 50 mM, from a 100 mM stock. Elution was allowed to take place for 120 minutes and removal of inhibitor on HA carried out as before.

Sample	Total activity (dpm x 10^6)	Recovery (%)
HA purified material	21.7 ± 1.62	100
Post affinity material	1.74 ± 0.16	8
Eluate	0.44 ± 0.04	2

Because of the very low recovery observed, nicotinamide was not used in further preparations.

4.3.15 Batch *versus* column affinity chromatography

Although batch chromatography had proved more successful than column chromatography in terms of recovery of enzyme activity, its use did, however, harbour a number of drawbacks.

Continued manipulation of the adsorbant, in particular the use of stirring bars, leads eventually to physical disintegration of the beads and hence poor flow rates when the adsorbant is loaded *via* a column. This is not a serious problem with small scale developmental work, although large scale preparations become increasingly difficult.

Continual transfer of gel material from columns to tubes and *vice versa* can also lead to gradual loss of adsorbant through binding to glassware, necessitating synthesis and characterisation of new affinity adsorbant.

Finally, washing and removal of non-interacting species is very difficult to achieve efficiently if adsorbants are loaded batchwise.

All of the above problems may be overcome if chromatography is carried out in columns, and accordingly effort was directed towards repeating the batch success with column chromatography.

Column chromatography

A 10 ml affinity column was loaded with 70 ml HA purified enzyme and washed with 50 ml SPB. 20 ml 20 mM 3AB in the same buffer was then applied to the top of the column, and after 3 ml had passed into the matrix (to minimise dilution), the outlet of the column was connected to the inlet *via* a peristaltic pump. The eluting buffer was recirculated at 30 ml/h for 90 minutes. Separation of eluted material and inhibitor was carried out as previously described and all fractions were assayed for enzyme activity.

Sample	Total activity (dpm x 10^6)	Recovery (%)
HA purified material	155	100
Post affinity material	3	1.9
Eluate	1.3	0.8

Recovery of enzyme activity was unacceptably low. This was probably due again to the problems of readsorption of eluted material to a highly substituted matrix. The concentration of competing ligand required for successful elution from the affinity

matrix therefore appeared to be higher for column systems than for batch.

Increased concentration of eluting inhibitor

As elution of the affinity adsorbant in column chromatography was unsuccessful with 20 mM 3AB, the concentration used successfully for batch elution, a 50% increase in the inhibitor concentration was tried.

A 2 ml affinity column, as opposed to the 10 ml column used previously, was used, due to the apparently high substitution of the support, to which was applied 75 ml HA purified material. The column was washed with 30 ml buffer and elution attempted as previously with 6 ml 30 mM 3AB in SPB. The eluting buffer was recirculated with a peristaltic pump to maximise desorption. Readsorption was minimised by connecting the outlet of the affinity column directly to the inlet of the 200 µl HA column. Eluted protein was therefore loaded directly onto the HA and could not react further with available ligand at the top of the column. After washing, the HA was eluted with 1 ml 0.6 M potassium phosphate buffer pH 8.0 and the fractions assayed in quadruplicate for enzyme activity.

Sample	Total activity (dpm x 10^8)	Recovery (%)
HA purified material	1.73 ± 0.16	100
Post affinity material	0.31 ± 0.01	18.4
Eluate	0.13 ± 0.01	7.5

Although only 7.5% of the applied enzyme activity was recovered (10% of bound material), the amount of acid insoluble radioactivity recorded in the affinity purified enzyme assays was very high

Sepharose itself could act as a gel permeation matrix. ∴ the low yield in columns vs batch elution could be due, entirely, to the effective removal of the inhibitor by the Sepharose at the top of the column, leaving little inhibitor for competition in the lower areas of the columns. This would be a much reduced problem in the batch elution

Total amount of Inhibitor — batch 0.03 m moles / ml of affinity ge
Column 1 0.04 m moles / ml of "
" 2 0.09 m moles / ml " "

relative to the previous assays. Indeed, 27% of the starting substrate was observed as TCA insoluble material and it therefore seemed probable that substrate depletion could account for the apparently low yields recorded.

4.3.16 Kinetic studies

Although v_{max} and K_m values are often quoted in the literature for ADPRT (Holtlund *et al.*, 1981; Agemori *et al.*, 1982; Zhang and Qiu, 1986; Petzold *et al.*, 1981), the figures should be treated with caution. The ADP-ribosylation system is particularly difficult to study due in part to the modulation of the system by a host of variable factors. As well as a requirement for Mg^{2+} ions and reduced thiol groups, the presence and nature of DNA, and type and availability of receptor molecules, is critical (section 1.3.1). These factors therefore contribute to a complex, finely balanced, system almost impossible to reproduce accurately from one laboratory to another.

For these reasons, v_{max} and K_m values for pig thymus enzyme purified by the method described were not determined.

Substrate depletion effects

Throughout development of the purification protocol, all enzyme activity determinations employed a substrate concentration of 500 nM, approximately two orders of magnitude below the recorded K_m of the enzyme.

Although the effect of substrate depletion will be less pronounced in crude extracts, the ratio of enzyme to substrate may increase substantially as purification proceeds.

Using a fixed lowered NAD^+ concentration, to pronounce any sub-saturating effects, varying amounts of nuclear extract were assayed and the relationship between enzyme concentration and product observed.

1 ml assays were set up in quadruplicate containing 5 µl NAD^+ (final concentration 250 nM), 2 mM DTT and varying amounts of freshly prepared salt extract of nuclei (non PEI-treated) from 5 to 975 µl. All assay volumes were made up to 1 ml with 0.1 M TEA.HCl pH 8.0 where necessary. After incubation of the tubes for 5 minutes at 26 °C, 250 µl ice cold 100% w/v TCA was added to each tube and precipitation and counting of radioactive product carried out (Figure 26).

It was noticeable that an increase in enzyme concentration up to 20 fold gave no significant deviation from linearity of product formation. However, between 20 and 100 fold increases, the amount of product formed almost levelled off. Further increase in the ratio led to almost no observed product with a 195 fold increase in concentration.

result of salt in the assay

If only one 'synthetic' enzyme species was present in the extract, observed product would/expect to level out into a plateau with increased enzyme addition. However, the salt extract is very heterogeneous and almost certainly also contains glycohydrolase. It was therefore difficult to resolve the contributions made to this deviation from linearity, by substrate depletion and product degradation.

Product observed over the standard 5 minutes assay period, instead of initial rate of product formation, also complicated interpretation, although previous studies had shown that the rate

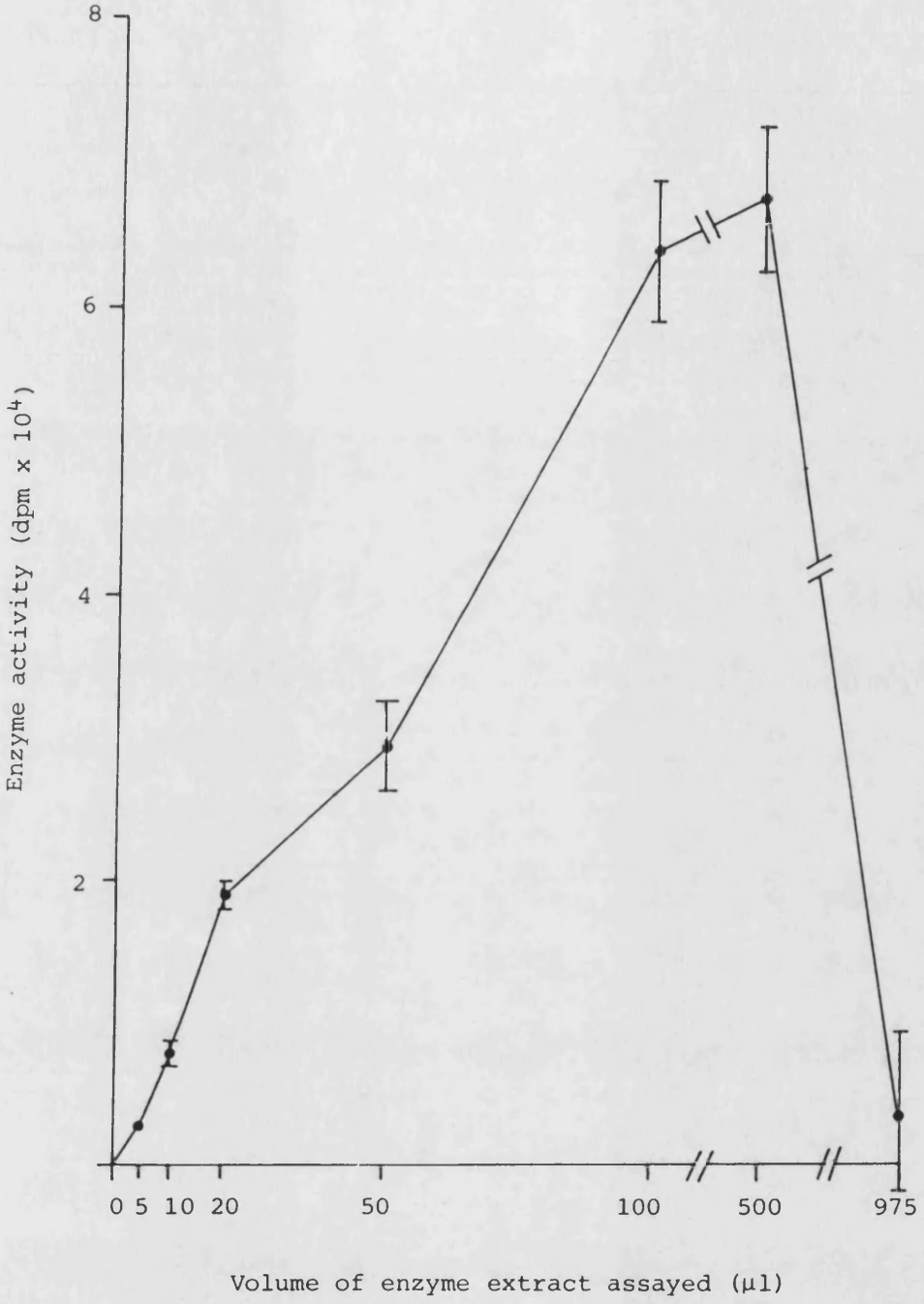


Figure 26 Effect of substrate depletion on TCA precipitable product.

Different volumes of nuclear extract (5-975 µl) were assayed for enzyme activity at a substrate concentration of 250 nM. Each point represents the mean of 5 determinations and the standard error of that mean.

of NAD^+ incorporation by nuclear extract was linear, at least up to 10 minutes.

Effect of substrate concentration on observed enzyme activity

Due to the difficulty in interpretation of the previous result, affinity purified enzyme was used. Approximately 2 µg enzyme was assayed at substrate concentrations below, above and approximately at the K_m of the enzyme (500 nM, 100 µM and 25 µM respectively) in quadruplicate.

The specific activity was varied to minimise waste using a stock solution of cold 2 mM NAD^+. The resulting activities were 2, 0.16 and 0.04 µCi/nmole for the 500 nM, 25 µM and 100 µM assays respectively (Figure 27).

Substrate concentration (µM)	Specific activity (nmoles/min/mg)
0.5	9
25	445
100	1313

Approximately 146 times more product was observed with a concentration of 100 µM than with 500 nM. This did not show a linear relationship reflecting the tendency towards saturation of enzyme with increasing substrate concentration.

To make any valid statement regarding yield of enzyme in any purification step, the concentration of substrate must be high enough not to be limiting. A concentration of 100 µM has been used almost universally in enzymological studies of ADPRT and was thus used to calculate the yield of the purification relative to the crude nuclear extract.

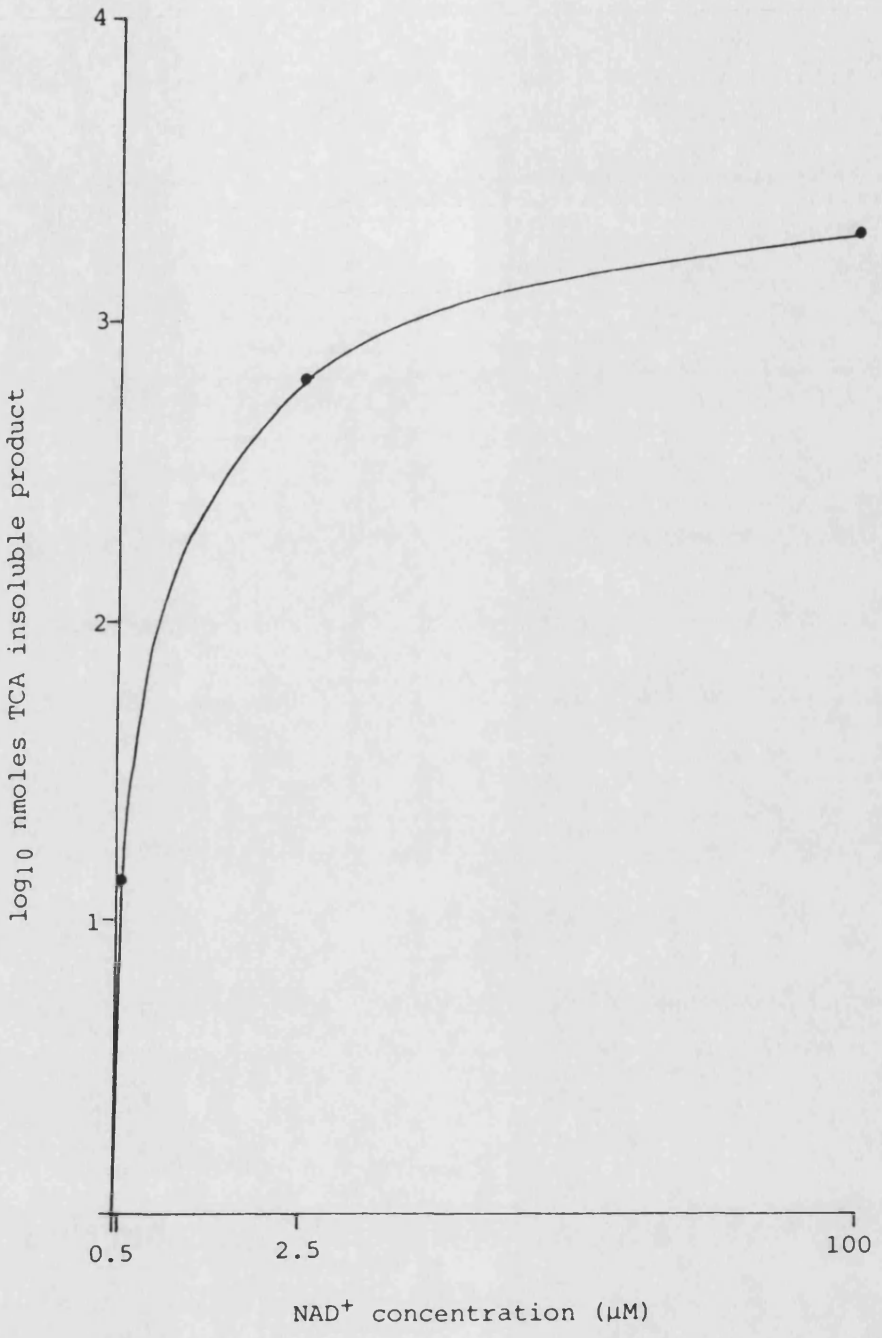


Figure 27 Effect of substrate concentration variation on TCA precipitable product generated by affinity purified enzyme.

4.3.17 Calculation of yield from purification protocol

The yield calculation was carried out on the purification protocol prior to development of the PEI extraction.

800 ml nuclear extract was diluted to 1600 ml with ice cold 0.1 M TEA.HCl pH 8.0. The extract was then treated with Cellex-QAE and HA in batches as described previously. The loaded HA was poured into a glass column, washed, and eluted stepwise with increasing concentrations of phosphate. The fractions containing enzyme activity (68 ml total) were mixed and loaded onto a 10 ml affinity column followed by overnight washing with 5 mM potassium phosphate pH 8.0. The NAD^+ specific activity was varied throughout with 0.32, 0.64, 0.12 and 0.01 μCi/nmole in the assays for nuclear extract, post Cellex-QAE, HA eluate and affinity purified enzyme respectively (results shown overleaf).

The specific activity of whole tissue is difficult to determine. However, if the literature value of Tsopanakis and co-workers (1978) is adopted, the overall figure for purification is (169.3 x 30.5) 5164 fold.

The largest loss of enzyme occurred with the HA step, where 67% of the net enzyme activity was accounted for, due largely to the disposal of the very small calcium phosphate HA fines.

Making the assumption that enzyme extracted from nuclei in the presence of PEI would behave in an identical manner to the extract used, less than 5% of activity would have been sacrificed at this stage. Correcting the appropriate figures in the table shown overleaf, the yield figures would thus have read as follows:

Stage of purification	Total enzyme activity (nmole/min)	Protein (mg)	Specific activity (nmole/min/mg)	Purification (fold)	Yield (%)
Nuclear extract	2706	912	3.0	1	100
Post Cellex-QAE	3476	560	6.2	2.1	128
HA eluate	888	89.8	9.8	3.3	33
Affinity purified enzyme	315	0.62	508	169.3	11.6

Stage of purification	Total enzyme activity (nmole/min)	Yield (%)
Nuclear extract	2706	100
HA	2571	95
Affinity preparation	912	34

A number of comparable specific activity values from the literature are given below.

Source	Specific activity (nmole/min/mg)	Reference
Calf thymus	1250	Ito *et al.*, (1979)
Calf thymus	3730	Zahradka and Ebisuzaki (1984)
Human placenta	1020	Ushiro *et al.*, (1987)
Sheep testis	1265	Zhang and Qiu (1986)

All of the above activities were higher than the value calculated for affinity purified pig thymus ADPRT. However, assay of a number of other affinity preparations showed the figure from section 4.3.16 to be more representative (1313 nmole/min/mg). The specific activity varied typically from around 1000 nmole/min/mg to about 1500 nmole/min/mg depending on the preparation.

4.3.18 Synthesis of a less substituted support

Use of 3AB as an eluting agent at a concentration of 30 mM would have proved prohibitively costly in the long run. Accordingly, a new, less substituted matrix was synthesised to facilitate elution with lower inhibitor concentrations. A reduction in substitution is also a possible means of lowering non-specific interactions.

Gel activation

Cyanogen bromide activation of 250 ml packed volume Sepharose-4B was carried out exactly as in section 4.3.5, except that 80 mg cyanogen bromide and 115 µmoles bisiminopropylamine per ml Sepharose were used, compared with 200 mg and 400 µmoles per ml respectively, in the previous activation.

Ligand coupling

Two coupling reactions were set up under identical conditions. The first, large scale, reaction consisted of 75 ml activated Sepharose in 75 ml DMF to which was coupled 1.0 g of succinylated 3AB using 1.5 g EDAC dissolved in 4 ml distilled H_2O.

The second reaction contained 10% of the amounts of constituents in the large scale coupling plus 0.625 µCi [^{14}C]-succinylated inhibitor. This reaction was set up in order to estimate the efficiency and level of coupling of ligand.

After the overnight incubation period, the gels were washed extensively and stored in 15% v/v ethanol.

Assessment of coupling efficiency

2 ml of gel coupled to radioactive ligand were diluted to a total volume of 10 ml with 15% v/v ethanol. Four aliquots of the suspension, of 1 ml each, were then counted for radioactivity in 9 ml Optiphase.

dpm per 0.2 ml packed Sepharose = 178 ± 12 (1)

dpm per 1 ml packed Sepharose = 890 (2)

µCi inhibitor coupled per ml $= \frac{890}{2.22 \times 10^6}$

$= 4 \times 10^{-4}$ (3)

$$\mu Ci \text{ inhibitor coupled per } 7.5 \text{ ml} = 7.5 \times 4 \times 10^{-4}$$
$$= 3 \times 10^{-3} \quad (4)$$

$$\text{Therefore \% coupled} = \frac{3 \times 10^{-3}}{0.625}$$
$$= \underline{0.5\%} \quad (5)$$

Calculation of ligand substitution

$$\text{Specific activity of uncoupled ligand} = 0.625\ \mu Ci/0.1\ g$$
$$= 1474\ \mu Ci/mole \quad (6)$$

From line (3) coupled radioactivity per ml $= 4 \times 10^{-4}\ \mu Ci$

$$\text{Therefore moles inhibitor coupled per ml} = \frac{4 \times 10^{-4}}{1474}$$
$$= 2.71 \times 10^{-7}$$
$$= \underline{0.3\ \mu moles} \quad (7)$$

4.3.19 Characterisation of new matrix

To determine the suitability of the new matrix for further use, the following experiment was carried out.

5 conical Pyrex tubes were set up on ice, tube 1 containing 0.5 ml SPB and tubes 2-5 containing 0.5 ml packed affinity matrix. 10 ml HA purified enzyme was added to each tube and mixing carried out by inversion for 30 minutes. The tubes were then spun for 30 s in a bench centrifuge to pellet the gel and the supernatant removed and assayed for unbound activity (8.1 ml was removed from tube 1 and 9.6 ml 50 mM 3AB added, followed by mixing). The gel containing tubes were washed twice with SPB before elution was attempted.

Elution was achieved by adding 3AB, in 2 ml volumes of buffer, to tubes 2-5 to give final concentrations of 1, 10, 20 and 40 mM

respectively. The tubes were mixed on ice over a 2 h period, after which the gel was pelleted and the supernatants, plus 2 ml of the contents of tube 1, assayed after separation from inhibitor on HA columns.

Tube No.	Unbound enzyme activity (dpm x 10^6)	Bound enzyme activity (dpm x 10^6)	Recovered enzyme activity (dpm x 10^6)	Recovery (%)
1 (Control)	11.82	-	9.72	82.2
2 (1 mM)	3.75	8.07	2.69	33.3
3 (10mM)	4.10	7.72	2.64	34.1
4 (20mM)	4.35	7.47	2.32	31.0
5 (40mM)	5.28	6.54	2.21	33.8

A minimum of 55% of available activity (tube 5) was bound by the new matrix, indicating that the adsorbant was capable of binding significant amounts of enzyme.

Recoveries were of similar size regardless of inhibitor concentration used, with approximately one third of bound enzyme recovery in all cases.

Large scale column chromatography

100 ml HA purified material was applied to a 20 ml affinity column *via* a peristaltic pump at a linear flow rate of 20 cm/h. The affinity column was then washed overnight with approximately 1 litre of 5 mM phosphate buffer pH 8.0. Elution was carried out with 10 ml 20 mM 3AB, recirculated in series with the HA column for 2 h. 20 mM 3AB was chosen to give a final concentration of approximately 10 mM allowing for dilution effects. 1 mM elution was not attempted due to the disparity observed in batch and column systems previously.

Sample	Enzyme activity (dpm x 10^8)	Recovery (%)
HA purified material	3.50	100
Post affinity material	0.06	1.7
Eluate	0.02	0.5

As stated previously, the apparent low recovery was primarily due to substrate depletion effects. 500 nM assays were used continually to detect enzyme activity, although total yields were calculated using enzyme saturating concentrations of substrate (section 4.3.16). 20% of NAD^+ in the assay was recovered as acid insoluble product.

A possible cause for concern was loss of activity due to proteolysis occurring during the wash stage. As salt extraction of the tissue, HA chromatography and affinity chromatography were all time consuming, the entire purification could not be carried out in only one day. The affinity column wash was therefore considered as an ideal opportunity to divide the purification into two sections. With such a long wash period, the possibility of activity loss had to be resolved.

HA purified enzyme was therefore assayed, after 18 h on ice in the presence of 25 mM sodium bisulphite, to determine any such losses. Only 13% of the activity in the crude preparation was lost over the time period chosen, and as column bound ADPRT would be essentially separated quite quickly from degrading species in the preparation, proteolysis did not present a big problem.

4.3.20 SDS-PAGE of affinity purified enzyme

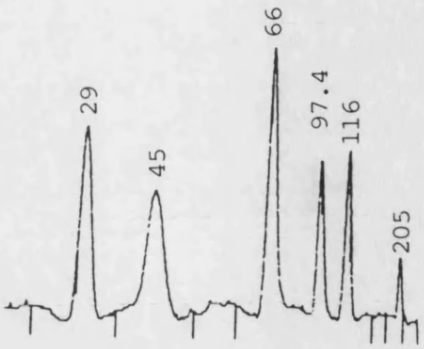
The affinity purified material from the previous section was concentrated to minimum volume in a Centricon 10 microconcentrator (Amicon). 2 ml distilled H_2O were mixed with the retentate,

followed by reduction to minimum volume again (∿40 µl) to reduce the potassium ion concentration. The retentate was then analysed on a 7.5% (w/v) polyacrylamide gel. After staining with Coomassie Blue, and destaining, as described previously, the gel was analysed by densitometry (Figure 28). From the scan it was obvious that a high number of contaminating species were still present, although in fairly small amounts. The peak areas, however, were not proportionate for all bands, due to the non-linear relationship between stain intensity and amount of protein present, ADPRT being by far the major constituent of the sample.

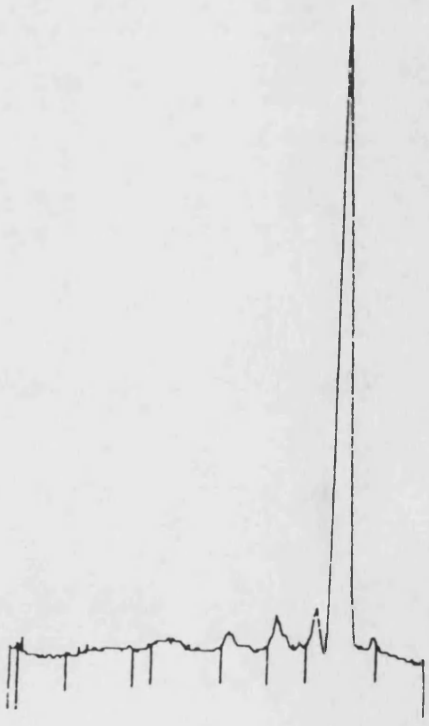
4.3.21 Hydrophobic interactions

Hydrophobic forces are probably the most important factor in aqueous enzyme-substrate interactions. The phenomenon has been defined broadly as "an interaction of molecules with each other which is stronger than the interaction of the separate molecules with water and which cannot be accounted for by covalent, electrostatic, hydrogen-bond, or charge-transfer forces" (Jencks, 1969).

The utilisation of such phenomena in protein separation was first realised in the early 1970's (Hofstee, 1973; Er-el *et al.*, 1972; Schaltiel and Er-el, 1973). Er-el and co-workers, working with a homologous series of alkyl agarose columns, observed that glycogen synthetase was retained by the substituted agaroses in an 'arm length' dependant manner. They noticed that the enzyme was excluded from columns containing 2 and 3 C atoms, but retained by a 4 C column, from which it could be recovered with a linear salt gradient. Higher members of the series bound the enzyme so tightly that it could not be recovered in an active form.



Molecular weight markers (Kd)



Affinity purified ADPRT

Figure 28 SDS-PAGE of affinity purified enzyme.

Electrophoresis was carried out as described in section 2.4. The gel was stained with Coomassie Blue and analysed by densitometry.

Such retention was recognised as the interaction of hydrophobic domains in the protein with the size-dependantly hydrophobic hydrocarbon arm.

Subsequently, interactions of this nature have been exploited with great success in fractionation of protein mixtures comprising species which differ significantly in the size and distribution of such apolar, hydrophobic pockets, a technique commonly termed hydrophobic affinity chromatography.

4.3.22 Removal of non-specific hydrophobic binding

For amino acid and sequence analysis of any protein, purity is of paramount importance. While ADPRT was undoubtedly the major constituent of the affinity purified material contaminating species had to be removed prior to such studies.

Such contamination was not surprising, however, when the nature of the affinity purification was considered. While attachment of the ligand itself conferred some hydrophobicity onto the support, reduction of the actual amount coupled proved inefficient at lowering non-specific interactions to acceptable levels (although easier recovery of enzyme activity was facilitated). The nature of this problem was probably augmented by the non-specific contribution made to the eluent by the use of 3AB. Another possible route for contamination was the presence of excess, uncoupled spacer arm on the support. Such hydrophobic extensions could represent sites for non ADPRT protein species to interact. Where this is the case, the problem may be overcome by attachment of the spacer arm to the ligand, prior to coupling to the support (Cuatrecasas, 1970a,b).

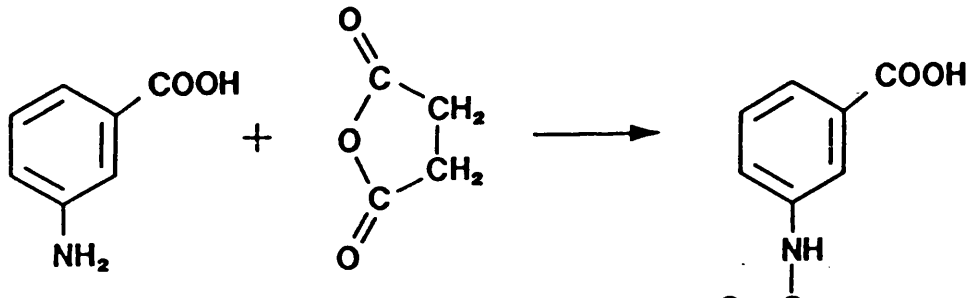
In an attempt to minimise such interactions, the possibility of using 3-aminobenzoic acid (3ABA), the acid analogue of 3AB as a competitive hydrophobic species was investigated. Such an approach can involve addition of the competitor in aqueous solution prior to chromatography, or in a wash step prior to elution. The method chosen, however, was to synthesise a screening column, whereby interfering species could interact prior to contact with the bio-specific adsorbant.

4.3.23 Preparation of a non-specific hydrophobic column

The similarity in structure of 3ABA and 3AB, and its relatively high K_i, made 3ABA a good choice for a competing species.

The only major drawback with its use was the presence of two carboxyl groups on the succinimidyl derivative, presenting two potential sites for attachment and the possibility of cross-linking.

Synthesis of 3-succinimidylbenzoic acid (3SBA)



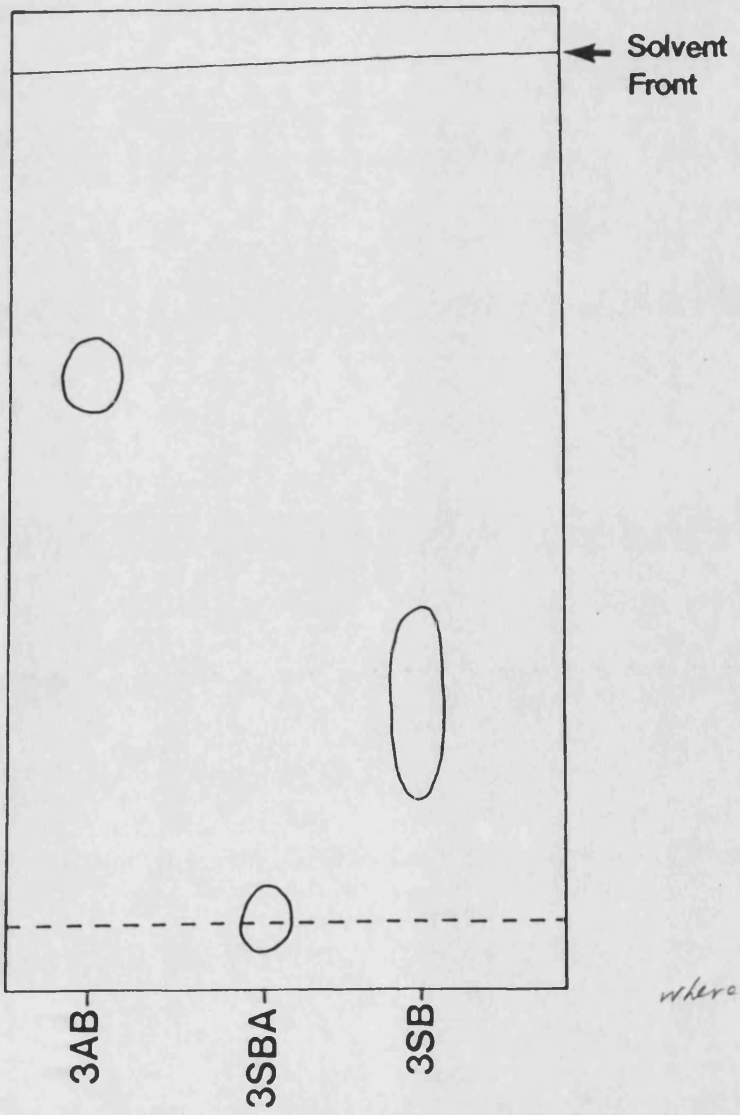
3ABA Succinic anhydride 3SBA

1.37 g (3ABA) was dissolved in 10 ml dry pyridine to which was added 1.1 g succinic anhydride, followed by mixing. The mixture was then left overnight at 20 °C. 10 ml distilled H_2O was added and the solution left for a further hour, to react

with any remaining succinic anhydride. The solvent was removed by rotary evaporation, followed by repeated addition and evaporation of 15 ml volumes of distilled H_2O. Succinic acid was removed from the mixture by selective precipitation and washing in distilled H_2O.

Product analysis

The product was dryed, followed by TLC analysis in the butanol system on a PEI plate. Migration was observed by placing the dried plate under ultraviolet light:



where is 3ABA

Only one spot was visible on the origin, which was expected with a dicarboxylic acid.

Coupling of product to activated Sepharose-4B

0.5 g product, dissolved in 10 ml DMF was added to 10 g, wet weight, activated Sepharose-4B (section 4.3.18), and coupling and washing carried out as previously.

Coupling of 3ABA to activated Sepharose-4B

Coupling was carried out as above, using 290 mg 3ABA.

Evaluation of binding to columns

1 ml columns of the three supports were poured and equilibrated in SPB. 14 ml HA purified enzyme was applied to each column, followed by 5 ml washes. The effluents and washes from each column were assayed for enzyme activity and protein (results shown overleaf). It was immediately apparent from the results that 3ABA coupled directly to the Sepharose-linker did not constitute a suitable non-specific adsorbant. 50% of applied activity was retained, accompanied by 16% of the applied protein. Such a loss of enzyme activity was unacceptable.

The succinimidyl derivative of 3ABA did, however, look promising. The apparent increase in enzyme activity alone, probably due to removal of glycohydrolase, was encouraging, as well as the 14% removal of protein, and constitutes a 1.3 fold purification in itself.

Sample	Enzyme activity (dpm x 10^6)	Enzyme recovery (%)	Protein (mg)	Removal of protein (%)
HA purified material	9.18	100	3.08	0
Post Sepharose-succinimidylbenzamide matrix	0.75	8	2.73	11
Post Sepharose-succinimidylbenzoic acid matrix	10.41	113	2.66	14
Post Sepharose-aminobenzoic acid matrix	4.63	50	2.59	16

4.3.24 Use of the 3SBA column as a final clean up step

The success of the previous experiment prompted further investigation of the derivatised support. However, affinity purified material, pre-processed by the non-specific support, showed no significant removal of contamination compared to non-3SBA processed material, when analysed by SDS-PAGE (result not shown).

The possibility of using the support as a final clean up step was therefore examined.

Four affinity purified ADPRT preparations were pooled and applied (with recirculation) by a peristaltic pump to a 3 ml 3SBA column, pre-equilibrated with 0.6 M potassium phosphate pH 8.0. ADPRT activity (x 4) and protein content (x 2), were determined before and after application. 5 µg of protein from the preparation before and after treatment was analysed by SDS-PAGE under reducing conditions. After Coomassie Blue staining and destaining, the gel was analysed by densitometry.

Sample	Enzyme activity (dpm x 10^8)	Protein (mg)
Pre-3SBA matrix	1.44 ± 0.05	2.56
Post-3SBA matrix	1.22 ± 0.05	2.25

A slight decrease in specific activity resulted from processing the material in the above manner. Observation of the gel scans showed no significant removal of contamination. Use of this support was therefore not pursued further.

4.3.25 Introduction of hydrophobic contamination by aliphatic spacer

To ensure that the bisiminopropylamine spacer was not introducing hydrophobic species to the preparation, the following experiment was carried out.

3 tubes (1-3) were set up on ice containing 1 ml affinity purified ADPRT each. To tube 1 was added 1 ml derivatised Sepharose (linker only) and to tube 2, 1 ml 3SBA-Sepharose. No addition was made to tube 3. The tubes were mixed gently, by regular inversion for 90 minutes, after which the gel was pelleted and the supernatants assayed for ADPRT. The supernatants were reduced to minimum volume in Centricon 10s and analysed by SDS-PAGE (Figure 29).

Sample	Enzyme activity (dpm x 10^5)
Post Sepharose-linker	9.6 ± 0.2
Post 3SBA matrix	10.3 ± 0.5
Affinity purified ADPRT	9.0 ± 0.3

No significant loss of activity was observed relative to tube 3. However, comparison of the samples after electrophoresis was only possible with tubes 1 and 2, as the contents of tube 3 were lost. The banding patterns of the samples were almost identical, however, and compared well with previous affinity purified ADPRT preparations. Whether material initially binding to and eluting from, the affinity adsorbant in a non-specific manner would display similar characteristics under the described conditions leads to complication of any interpretation.

It was concluded, however, that the presence of any free bisiminopropylamine was not responsible for introducing the large degree of

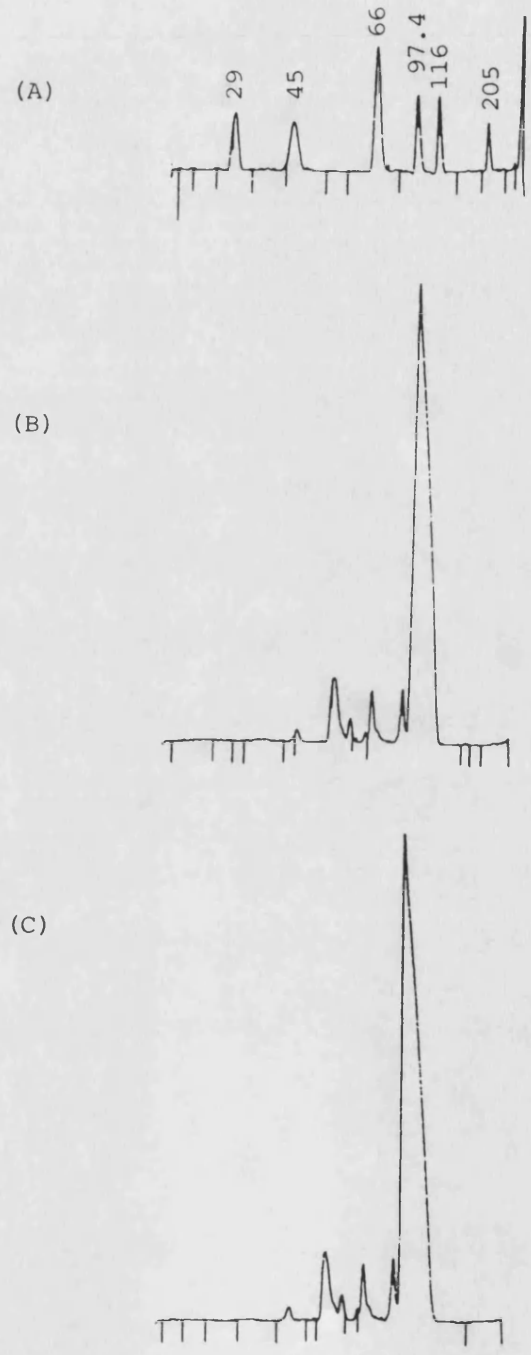


Figure 29 Evaluation of contributions made to binding by the bis-iminopropylamine spacer arm.

Affinity purified material was processed with Sepharose-bisiminopropylamine before (B) and after (C) ligand had been coupled. The unbound material was concentrated and resolved by SDS-PAGE followed by gel densitometry.

Scan (A) represents molecular weight markers which are quoted in Kd (see text for details).

contamination observed. Hence other methods of further purification were pursued.

CHAPTER 5

Further Purification and Enzyme Characterisation

5.1 Attempts at Further Purification

5.1.1 Gradient elution of affinity purified ADPRT from final HA column

Pooling the enzyme rich fractions from the preparative HA stage may have been responsible for the inclusion of hydrophobic contaminants eluted towards the beginning and end of the ADPRT peak. Gradient elution of the final HA column was carried out in an effort to resolve these components.

85 ml HA purified material was loaded onto an affinity column and eluted as previously in series with a HA column. After washing, the HA column was eluted with a linear, 50 ml, 0.2-0.6 M potassium phosphate buffer gradient. 23 fractions of approximately 2 ml each were collected and assayed for protein and enzyme activity (Figure 30).

Enzyme was eluted over a large concentration range, from 263 to 528 mM, and subsequent analysis of fractions at the front, middle and end of the enzyme peak by SDS-PAGE showed no significant purification.

5.1.2 Non-dissociating acid PAGE

The band pattern observed for affinity purified ADPRT after SDS-PAGE was very similar with each preparation. The three major contaminating species observed were always present at approximately 92, 66 and 43 Kd. Such size differences made gel filtration an appealing candidate for further purification.

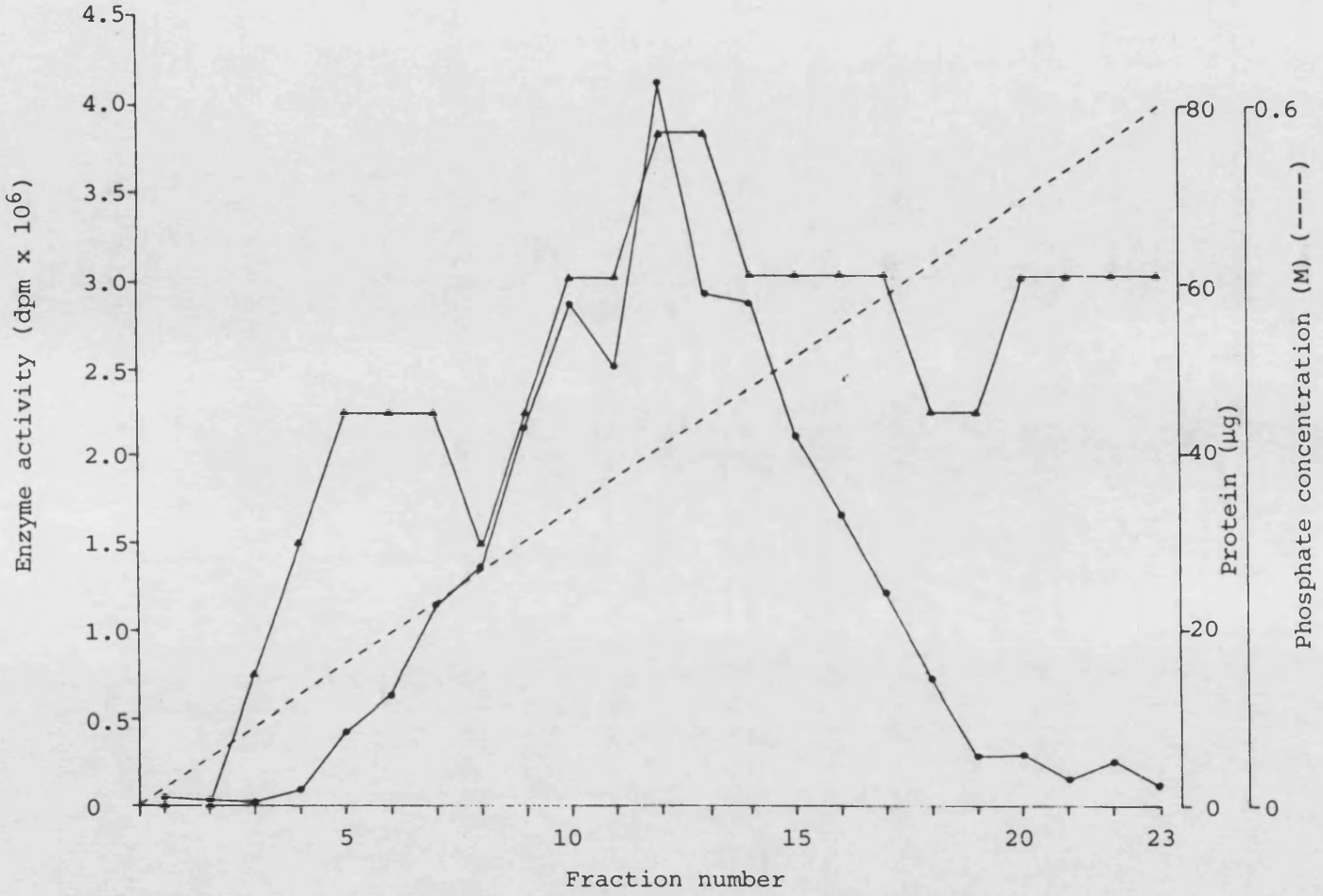


Figure 30 Gradient elution of second HA column.

Affinity purified enzyme was eluted directly onto a 0.5 ml HA column which was subsequently eluted with a linear phosphate gradient (0.2-0.6 M).

Each fraction (∿2 ml) was assayed for enzyme activity (●) and protein content (▲).

Electrophoresis under non-dissociating conditions often gives a good indication of a protein's behaviour in gel filtration chromatography and was thus carried out on affinity purified ADPRT.

An acid system was chosen (Hames and Rickwood, 1981) as ADPRT is reported to have a high P_i and hence carries a positive charge at low pH. 5 µg of affinity purified ADPRT was electrophoresed in a system which stacked at pH 5 and resolved at pH 3.8 using methylene blue as tracking dye. On staining, resolution was observed to be very poor, exhibiting smearing throughout the stacking gel and on the top of the resolving gel (Plate 2).

Although this was not encouraging, gel filtration was pursued, in the hope that better separation might be achieved with the different conditions employed.

5.1.3 Gel filtration of affinity purified ADPRT

The cross-linked polyacrylamide medium, BioGel-P150 (BioRad), was chosen, which has an exclusion limit of approximately 150 Kd. The gel was prepared according to the manufacturers instructions and a 30 x 1.5 cm column poured. The column was equilibrated in SPB and the void volume determined as previously (section 3.5). 200 µl affinity purified material (1 mg/ml) was applied to the top of the column and chromatography carried out with a constant flow rate of 7 cm/h. 60 fractions of approximately 0.6 ml were collected and assayed for protein and enzyme activity (Figure 31).

90% of the protein and greater than 95% of the enzyme activity applied was collected immediately post void volume, reinforcing the observation with non-denaturing PAGE. As the

PLATE 2 Non-denaturing PAGE analysis of 5 µg affinity purified ADPRT

Plate 2 Non-denaturing PAGE analysis of 5 µg affinity purified ADPRT (see text for details).

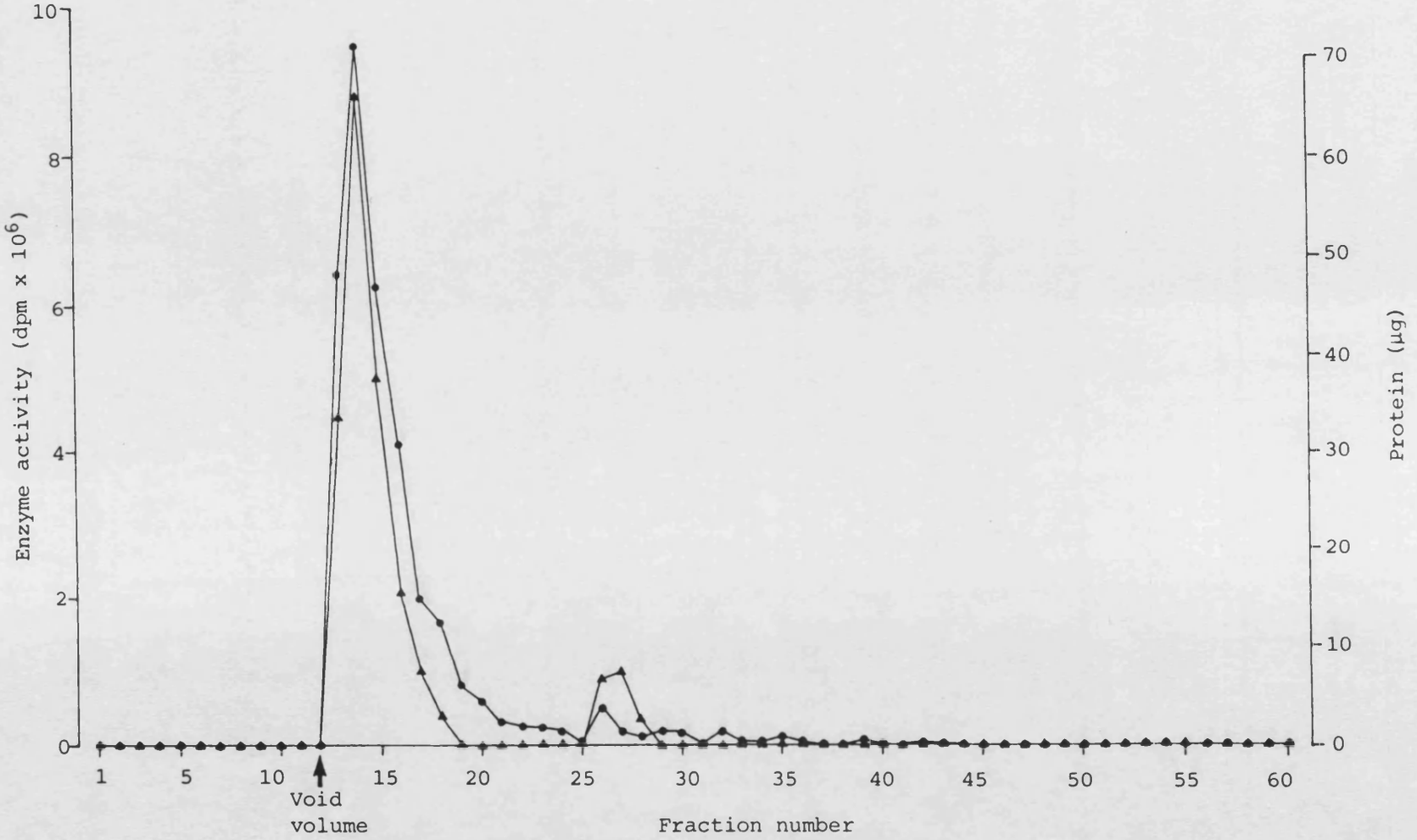


Figure 31 BioGel P150 chromatography of affinity purified ADPRT. 200 µg affinity purified enzyme was chromatographed on a 30 x 1.5 cm column (see text for details). All fractions (0.6 ml) were assayed for enzyme activity (●) and protein content (▲).

major component of the mixture was 116 Kd in size, this indicated aggregation of components, leading to exclusion from the matrix. A small proportion of material did appear to be included, but this represented only 2.5% of the applied activity.

To confirm this result, fractions 13 to 16 and 26 to 28 inclusive, were concentrated by ultrafiltration and analysed by SDS-PAGE. Although the included fraction showed no visible bands after staining, possibly due to non-specific binding to the filtration membrane, the excluded fraction exhibited similar band distribution to untreated affinity purified ADPRT.

Gel filtration was therefore not pursued as a means of further purification.

5.1.4 Phosphocellulose chromatography

While salt extraction of chromatin is analogous to ion-exchange, an ion-exchange step 'proper' had not been successfully incorporated into the purification protocol thus far. A number of literature protocols include the cation exchanger phosphocellulose (Okazaki *et al.*, 1975; Ushiro *et al.*, 1987; Burtscher *et al.*, 1986), surprisingly quite often as a final clean up step (Zhang and Qiu, 1986; Okayama *et al.*, 1977; Mandel *et al.*, 1977).

Although affinity purified enzyme did bind phosphocellulose in 5 mM potassium phosphate buffer, no further purification was obtained, as enzyme activity was recovered immediately after elution was attempted, both with increased phosphate and KCl concentrations. Whether this was due to the presence of small amounts of residual DNA or to the use of phosphate buffer at

pH 8.0, or both, was difficult to assess, therefore other methods were sought.

5.1.5 Hydrophobic affinity chromatography

As the contaminants present in the affinity purified material had previously interacted positively with an essentially hydrophobic medium, the non-specific support Phenyl-Sepharose CL-4B (Pharmacia) was employed in further efforts to separate the components of the mixture by virtue of potential differences in hydrophobicities.

A 0.5 ml column was poured and equilibrated with 0.6 M potassium phosphate buffer pH 8.0, 3.0 ml affinity purified ADPRT was loaded onto the column, which was then washed with 5 ml 0.6 M potassium phosphate. Elution was carried out with a 45 ml, linear 0-75% v/v ethylene glycol gradient. 47 fractions were collected and assayed for enzyme activity and protein content (Figure 32).

On initial examination, the elution profile looked very promising. However, the enzyme activity peak was accompanied by only 43% (230 µg) of recovered protein. SDS-PAGE had previously shown that one band migrating with an approximate molecular weight of 116 Kd was the major constituent in the preparation, and it was therefore surprising that the proportion of protein associated with the enzyme peak was so small.

SDS-PAGE analysis of the enzyme peak, however, still showed contaminating material migrating with sizes corresponding to 92, 66 and 43 Kd, plus a band of approximately 54 Kd. The possibility of the enzyme being particularly labile, due to

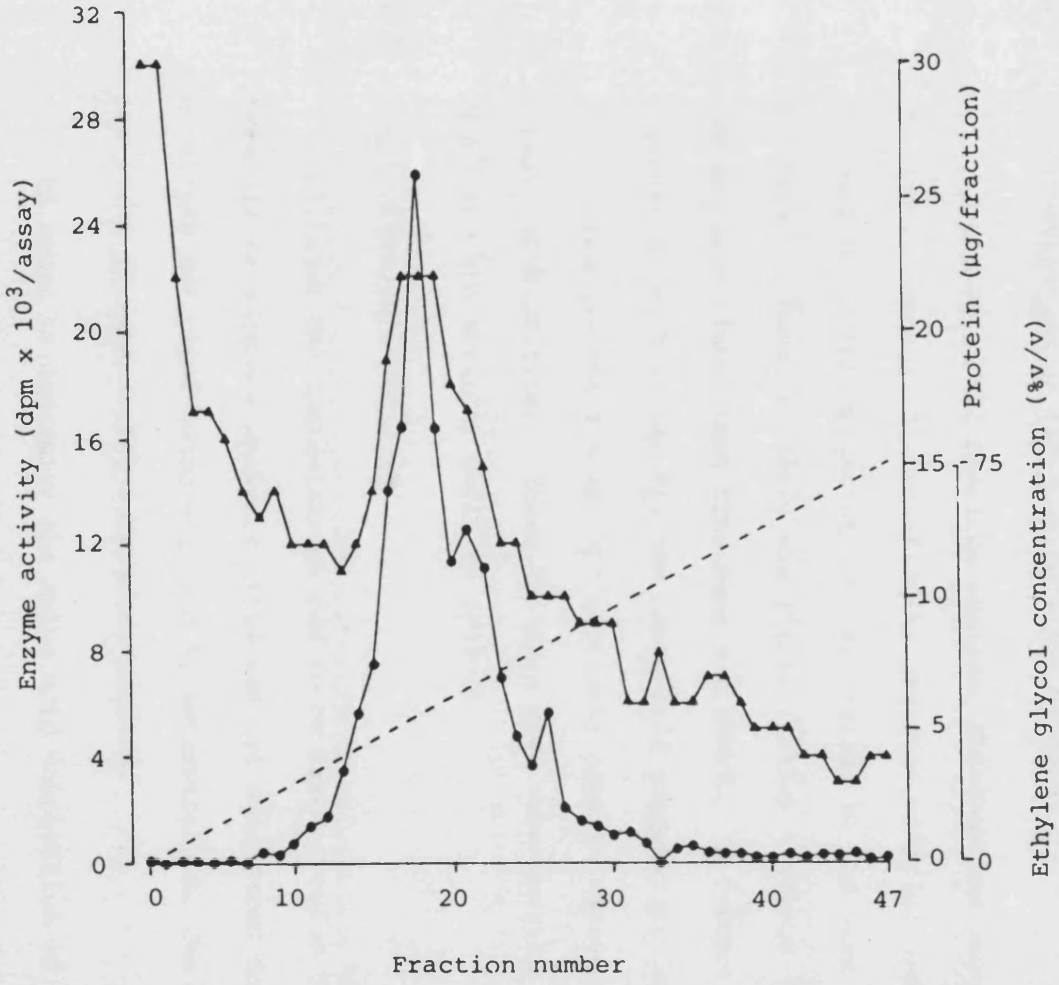


Figure 32 Hydrophobic affinity chromatography of affinity purified ADRPT.

540 µg affinity purified material was applied to a 0.5 ml Phenyl Sepharose column and elution carried out with a 45 ml ethylene glycol gradient (0-75% v/v).

All fractions (~1 ml) were assayed for enzyme activity (●) and protein content (▲).

either chemical instability or copurification of proteases, and the smaller contaminants being merely fragments of the native enzyme was not discounted at this stage. The bands of approximately 54 and 66 Kd, for example, could have been the result of a single cleavage in the native polypeptide.

5.2 Characterisation of Affinity Purified Material

As stated in the previous section, SDS-PAGE had shown that the major component of the affinity purified material had an apparent molecular weight of 116 Kd, similar to the size recorded for ADPRT. However, there was little further evidence to substantiate claims that this band was ADPRT. A number of parameters, such as the P_i, the amino acid composition and type of NH_2-terminal amino acid, are routinely used to characterise a particular protein. These features were subsequently determined for the affinity purified protein.

5.2.1 Total amino acid analysis

Although the contamination had to be removed prior to NH_2-terminal or sequence analysis, this was not considered to be necessary for total amino acid and P_i determination, due to the low level of interfering substances present.

In order to determine the amino acid composition of a polypeptide, the amino acid components must first be liberated from the molecule. This is done by hydrolysis in 6N HCl at 110 °C for at least 24 h. The hydrolysate is then loaded onto an ion-exchange column, usually a sulphonated polystyrene resin such as Dowex-50, and eluted with a gradient of increasing pH. The bound amino acids are desorbed in order of increasing basicity with aspartic acid eluting first and arginine last.

The equivalent of 30 µg of protein was spotted onto the bottom of each of six Pyrex hydrolysis tubes and the solvent removed in a vacuum dessicator. 200 µl degassed 6N HCl, containing 0.5 µl/ml β-ME to protect methionine residues, was added and the samples frozen in dry ice. The tubes were then evacuated and the contents allowed to melt. Once production of bubbles had stopped for 3 minutes, the tubes were sealed using an O_2/gas torch. The tubes were then incubated at 105 °C, in pairs, for 24, 36 and 72 h.

At the end of the hydrolysis period, the tubes were allowed to cool, opened, and the solvent removed, again in a vacuum dessicator. The amino acids were dissolved in 20 mM HCl, containing norleucine as an internal standard, and the components separated on a Rank Hilger, Chromaspec II amino acid analyser.

Amino acid composition

	(%)
Lys	12.1
His	1.9
Arg	.3.1
Asp	9.3
Thr	3.8
Ser	7.6
Glu	13.3
Pro	7.1
Gly	6.5
Ala	8.3
Val	5.9
Met	2.5
Ile	3.9
Leu	8.6
Tyr	3.5
Phe	2.9

The value for Glu and Ala were slightly high relative to literature values and Gly slightly low, otherwise the figures agreed quite well. (Pro was also quite high, but may be an inaccurate estimate, as it was calculated from the relative weight of the printed trace from the amino acid analyser.)

5.2.2 P_i determination

The charge on any protein at a given pH value is the net product of the total charge held by the side chains of the constituent amino acids and the terminal carboxy and amino residues.

In isoelectric focussing (IEF), a protein (or proteins) is electrophoresed in a support carrying a stable pH gradient. The protein migrates in the field until it reaches the point in the gradient at which its net surface charge is zero. This pH is termed the P_i of the protein.

The pH gradient is established by inclusion of a solution of low molecular weight molecules called carrier ampholytes. These molecules have very high buffering capacity at their own P_i values and low buffering capacity around their P_is. In an electric field, they migrate through the support and arrange themselves in order of increasing P_i from anode to cathode.

IEF is carried out in a number of insoluble supports of which polyacrylamide slabs or rods seem most popular. It is the highest resolution electrophoretic technique available for protein separation, being capable of separating proteins differing by only 0.001 pH unit in P_i.

ADPRT is quite a basic molecule with literature P_i values in most cases from pH 9-10 (Ito *et al.*, 1979; Petzold *et al.*, 1981; Kristensen and Holtlund, 1980; Ushiro *et al.*, 1987). The P_i of approximately 1 µg of affinity purified material was determined in triplicate, by the following method:-

Solution A (100 ml), filtered before use

14.25 g	Acrylamide
0.75 g	Methylene bisacrylamide
25.00 g	Sucrose

Solution B (100 ml), stored in dark bottle

2 mg	Riboflavin
60 mg	Potassium persulphate

Solution C (sufficient for 12 rod gels, 10 x 0.5 cm i.d.)

9.6 g	Analar urea (BDH)
10 ml	Solution A
1 ml	Ampholine pH 3-10 (Pharmacia)
4 ml	Distilled H_2O

— why a narrower basic range ampholine not used

After mixing, 5 ml solution B and 40 µl TEMED were added, followed by degassing.

Glass rods (12 x 0.5 cm i.d.) were washed in concentrated HCl for two days prior to use and stored in distilled H_2O until required.

The cleaned tubes were rinsed in 95% v/v ethanol and dried in a stream of warm air. The bottoms of the tubes were capped with parafilm and clamped in an upright position and 2 ml solution C added to each tube with a long form pasteur pipette. Distilled H_2O was then layered onto the top of the gels to aid polymerisation and the gels allowed to set (approximately 1 h). The parafilm

caps were then removed, excess distilled H_2O removed with blotting paper and the tubes placed in the electrophoresis apparatus. 50 µl protection buffer (2.5% v/v sucrose, 5% v/v Ampholine) was layered onto the gel surfaces and the tubes topped up with catholyte (20 mM NaOH). The remaining catholyte and the anolyte (10 mM H_3PO_4) were added to the appropriate reservoirs, the cooling water jacket switched on and the gels electrophoresed at 200 V for 1 h to prefocus the ampholytes. After prefocussing, the catholyte was decanted off and buffer removed from the tops of the tubes as previously. The sample was layered onto the top of the gels followed by 50 µl protection buffer, the catholyte replaced and electrophoresis carried out for 20 h at 500 V.

After running, the gels were removed from the tubes by rimming and placed in clean tubes. Fixer (15% w/v TCA, 5% w/v sulphosalicylic acid) was added to fix the protein and remove ampholytes (which can interfere with some staining methods), and the tubes left for 1 h at room temperature covered in foil. The fixed gels were immersed in stain (0.084% v/v Coomassie Brilliant Blue R-250, 10.8% w/v TCA, 3.24% w/v sulphosalicylic acid, 27% v/v methanol) for 3 h at room temperature and then destained with successive volumes of destaining solution (10% v/v ethyl acetate, 5% v/v glacial acetic acid, 7% v/v methanol). A blank tube was also run to measure the pH gradient which was determined thus:

Immediately post electrophoresis, the blank gel was cut into 0.5 cm lengths on a clean glass plate with a scalpel. The lengths of gel were soaked in 1 ml KCl (25 mM) for 1 h at 4 °C before measuring the pH of each slice with a standard pH probe.

The pH measurements did not reflect establishment of a smooth pH gradient from 3 to 10. Although up to pH 6.5 a smooth increase was observed, above this, pH quickly levelled off to about pH 7.5.

Increasing pH, however, facilitates increased CO_2 solubility. The apparently low values observed were doubtless due to titration of the experimental pH levels to the observed values by atmospheric CO_2 during the pH measurement period. In some experimental cases, this phenomenon can lead to severe pH drift, although it may be overcome by displacing CO_2 from the system with N_2 gas. In the system described, however, such problems were hopefully minimised, due to the low levels of contact with the atmosphere accorded by the rod gel method.

To make P_i measurements of proteins in alkaline gradients, it is therefore advisable to include proteins of known P_i as markers.

The gels containing sample all exhibited one band approximately 1-2 mm from the top of the gel (Plate 3). If the problem of pH drift had been evaded successfully and a linear gradient of pH 3-10 established, the P_i of the main constituent of the affinity preparation was approximately pH 9.5-10, which corresponds well with quoted literature values.

5.3 Preparative SDS-PAGE

Use of affinity purified material for NH_2-terminal amino acid analysis and sequencing studies was not contemplated, due to the possibility of contaminating species being present in

PLATE 3 Isoelectric focussing of affinity purified ADPRT

Plate 3 Isoelectric focussing of affinity purified ADPRT (1 µg) (see text for details).

high enough quantities to give ambiguous results. NH_2-terminal amino acid analysis by the dansyl method requires at least nanomolar levels of peptide for detection. With a molecule the size of ADPRT, this corresponds to hundreds of micrograms. As the affinity purified material was not entirely homogenous, the presence of nanomolar quantities of much smaller peptides could not be discounted.

In overloaded gels, previously studied for contamination, it was observed that migration of the major component of the mixture could be followed quite easily due to visible differences in the refractive indices of polyacrylamide gel containing, and in the absence of, protein. This feature allowed removal of polyacrylamide sections containing only the 116 Kd constituent, from the gel slab.

5.3.1 Two methods of passive elution from polyacrylamide gel

The potential of passive elution using finely and coarsely divided polyacrylamide gel pieces was assessed.

Sakakibara and co-workers (1987) reported 90-100% recovery of BSA from agarose gels using an extraction method involving homogenisation and centrifugation. The applicability of this method to protein separated in a 7.5% v/v SDS-PAGE system was determined as follows.

After electrophoresis of 775 µg affinity purified material, the major band was excised and cut into two equal pieces, A and B, with a clean scalpel.

Piece A was placed in a clean Corex tube containing 3 ml PBS and homogenisation carried out at full speed using an Ultra-Turrax probe (Ika-Werk, Jank and Kunkel Laboratory, Sweden). The tube was then centrifuged at 5000 g for 10 minutes, the supernatant removed and kept on ice. The extraction step was repeated twice, followed by concentration of each extract to suitable volumes for SDS-PAGE analysis (Plate 4A).

Piece B was placed in a 5 ml polystyrene Sterilin tube containing 3 ml 0.1 M ammonium acetate buffer pH 8.0. The tube was placed on a shaking water bath for 24 h, after which the buffer was removed and concentrated for SDS-PAGE analysis as above (Plate 4B).

The quantities of protein recovered are difficult to assess, due to interference by SDS and Tris in the dye binding and Lowry assays respectively. However, it was quite obvious that homogenisation of polyacrylamide, containing the protein species of interest, resulted in severe fragmentation of the polypeptide.

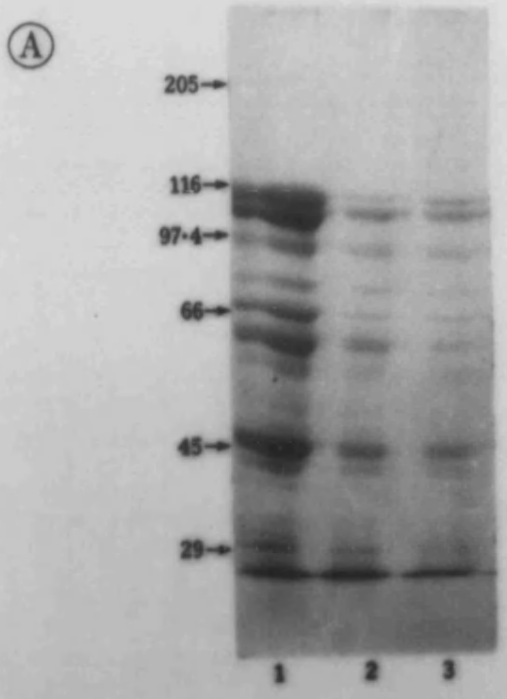
Agarose gel is held together by weak non-covalent, hydrogen-bonding, whereas covalent cross-linking is responsible for the structure of polyacrylamide. Not surprisingly, agarose gel is easier to disrupt. The relative ease with which agarose gel can be cut cleanly, relative to the often uneven tearing of polyacrylamide gel, and the reversibility of agarose gel formation clearly demonstrates this difference.

While division of the gel into fine particles gives greater surface area for elution, it would appear that with such a large protein, disruption of the cross-linked acrylamide gel resulted

PLATE 4 Passive elution of preparative SDS-polyacrylamide gels. Polyacrylamide gel pieces containing equal quantities of ADPRT were eluted as follows, prior to SDS-PAGE analysis:

(a) The gel piece was homogenised in PBS, followed by centrifugation and concentration of the supernatant. Lanes 1, 2 and 3 represent the first, second and third extractions respectively.

(b) The intact gel piece was extracted by gentle agitation in 3 ml 0.1 M ammonium acetate buffer pH 8.0, followed by concentration of the extract (lane 2). Lane 1 shows standard high molecular weight markers.


A
205
116
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1
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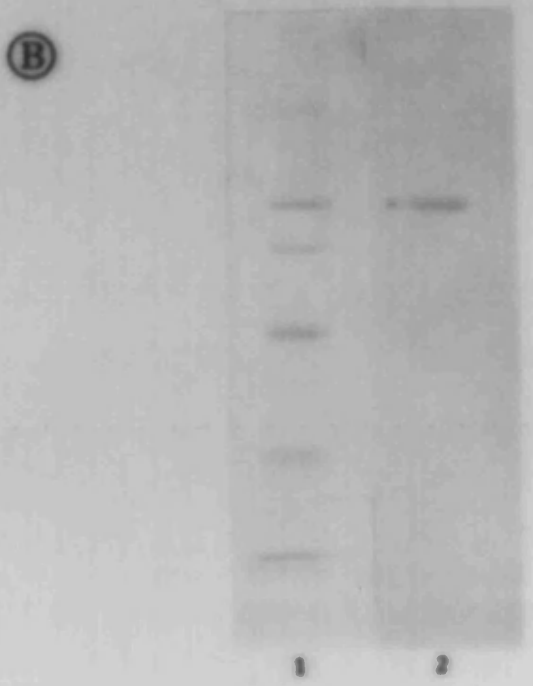

B
1
2

in physical stress and breakage of the polypeptide.

Employment of gentle, passive diffusion with the whole gel piece was far more satisfactory in terms of the homogeneity of recovered protein. However, from gel analysis alone the yield observed was very low. Surprisingly, the presence of contaminating bands, similar to contamination observed previously, was also noticed, albeit to a lesser extent, adding weight to the argument that lability of the native protein, when subjected to SDS treatment at high temperatures, was partly responsible for the heterogenous nature of banding observed (Weber *et al.*, 1972). Fragmentation of purified ADPRT by similar SDS treatment was also observed by Ushiro and co-workers (1987) and accordingly, the pattern of bands obtained from gel piece B was regarded as that produced from a purified enzyme preparation.

Clearly, the extraction method described for gel piece A could not be applied to polyacrylamide gel in this instance. Passive diffusion as described for gel piece B was more successful, but was limited in its application at this stage, due to the low yield observed.

5.3.2 Electroelution from polyacrylamide gel

Electroelution is probably the most popular method employed for quantitative recovery of protein from polyacrylamide gel. In most methods, the gel piece is finely divided and embedded in tubes of agarose or low concentration polyacrylamide. Application of a potential difference across the gel results in migration of the protein towards the anode (in the case of SDS-PAGE) allowing recovery as it leaves the gel. A variety of

collection methods have been used successfully. Attachment of dialysis tubing to the appropriate end of the gel tube and adsorption of eluted protein onto HA plugs are two such methods.

In this case, the means of recovery chosen involved collection of a stream of reservoir buffer which had been passed across the surface of the gel during electrophoresis. This was achieved with the use of the apparatus shown diagrammatically in Figure 33.

Approximately 0.75 mg affinity purified ADPRT was separated on a 7.5% w/v polyacrylamide gel using the Laemmli buffer system. Immediately post-electrophoresis, the major band was excised and placed in a sterile 5 ml syringe. The gel was then broken by expulsion through the syringe tip into a glass gel rod of 1 cm internal diameter, sealed at the bottom with parafilm. 3.5 ml polyacrylamide (3.75% w/v pH 8.8) was added to the tube through the syringe and the mixture agitated to remove air bubbles. Distilled H_2O was layered over the top of the gel and the tube left to set for 1 h. The tube was placed in the electrophoresis apparatus and the reservoirs filled with standard reservoir buffer (192 mM glycine, 25 mM Tris, 1% w/v SDS). The peristaltic pump was switched on and electrophoresis carried out for 3 h at 300 V. Ten fractions of 1 ml were collected and the OD_{280} measured for each. Although SDS interferes at this wavelength, a clear peak was visible and such measurements were considered a suitable means of determining the end of protein elution (Figure 34). The fractions were dried overnight by vacuum dessication and the contents of each tube dissolved

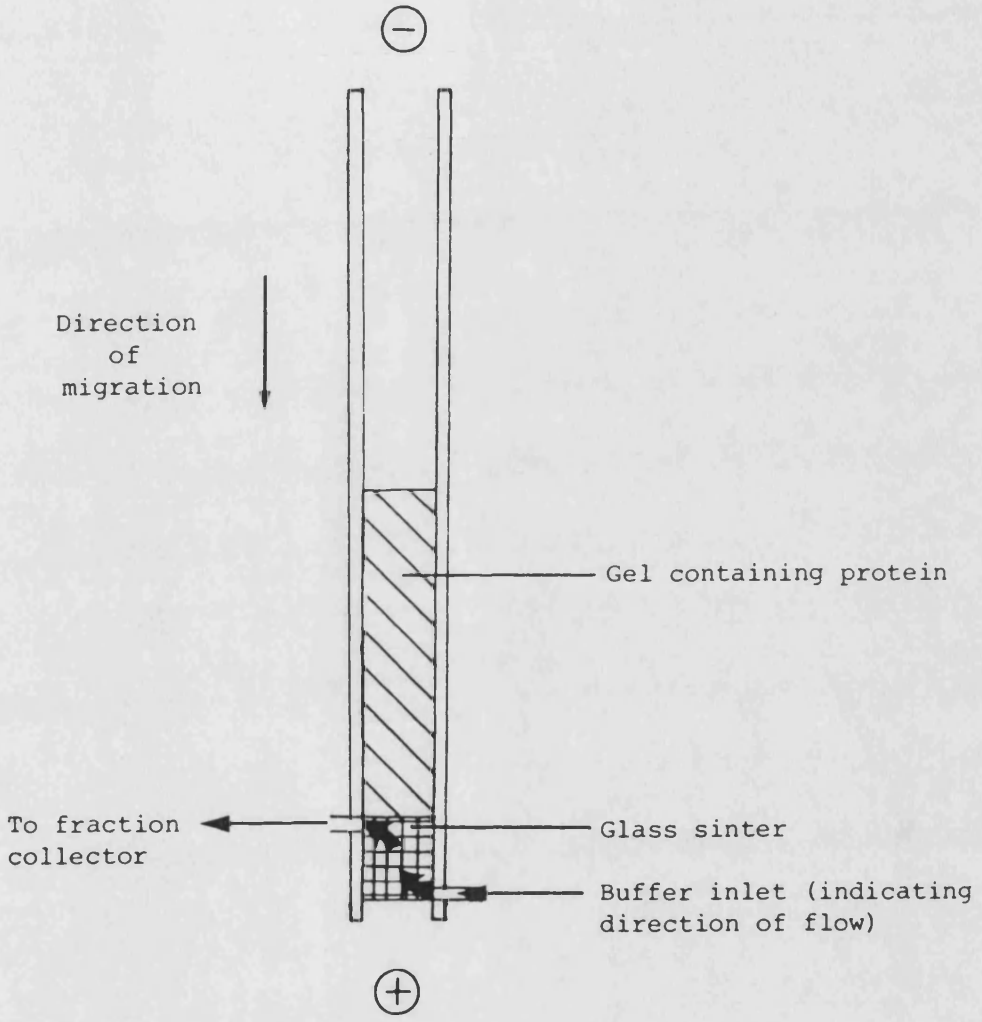


Figure 33 Diagrammatic representation of electroelution apparatus.

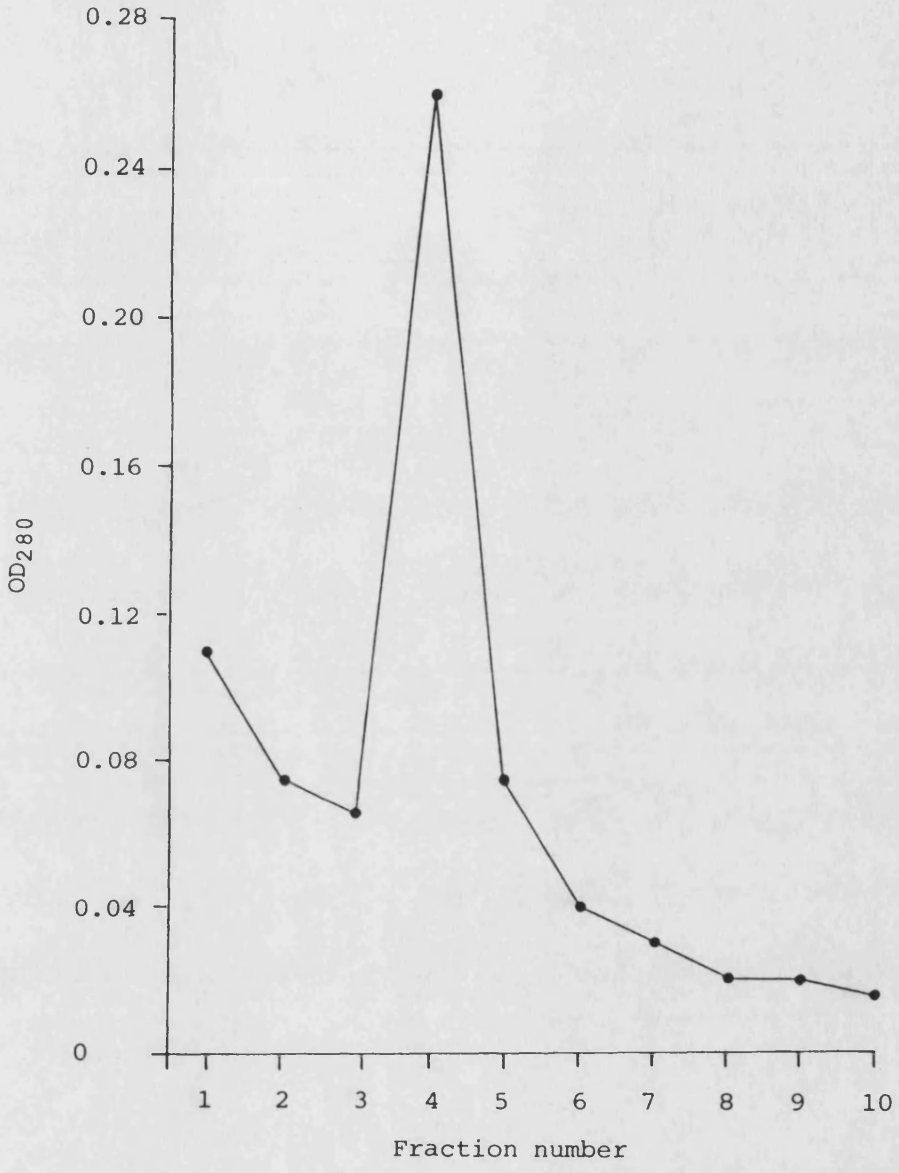


Figure 34 Electroelution of ADPRT.

0.75 mg affinity purified ADPRT was resolved by SDS-PAGE using the buffer system of Laemmli (1970). Following excision of the ADPRT containing gel, elution was carried out as described in the text.

in 100 µl distilled H_2O. 5 µl of each fraction was analysed by SDS-PAGE (7.5% w/v) and silver stained (Plate 5).

The gel showed one protein band at approximately 116 Kd, in each of fractions 1-5. The smaller bands of 50-60 Kd are artifactual and arise when β-ME is employed to reduce disulphide bonds.

Material eluted in this way, however, was not suitable for amino acid analyses, due to the presence of residual glycine from the electrophoresis buffer system. Further electro-elution studies were carried out utilising buffer systems with non-interfering components.

5.3.3 Preparative SDS-PAGE using a phosphate buffer system

While resolution is not as good as that attainable with the Laemmli buffer system, the electrophoresis system developed by Weber and Osborn (1969; Weber *et al.*, 1972) was similarly used to attempt recovery of homogenous protein as in the previous section.

Separation was carried out in a 7.5% w/v polyacrylamide slab with a 3.5% w/v stacking gel. The resolving gel was made by mixing 18.5 ml SDS-phosphate buffer (0.2 M sodium phosphate buffer pH 7.2 containing 0.2% w/v SDS), 10 ml stock acrylamide (% T = 22.8, % C = 2.6) and 45 µl TEMED in a Büchner flask. After degassing, 1.5 ml fresh 1% w/v ammonium persulphate solution was quickly mixed with the solution and the gel cast. When set, the stacking gel was poured, consisting of 15 ml SDS-phosphate buffer, 3 ml stock acrylamide, 45 µl TEMED and 1.5 ml ammonium persulphate, prepared as for the resolving gel.

PLATE 5 SDS-PAGE analysis of material electroeluted from 'Laemmli' preparative SDS-polyacrylamide gel.

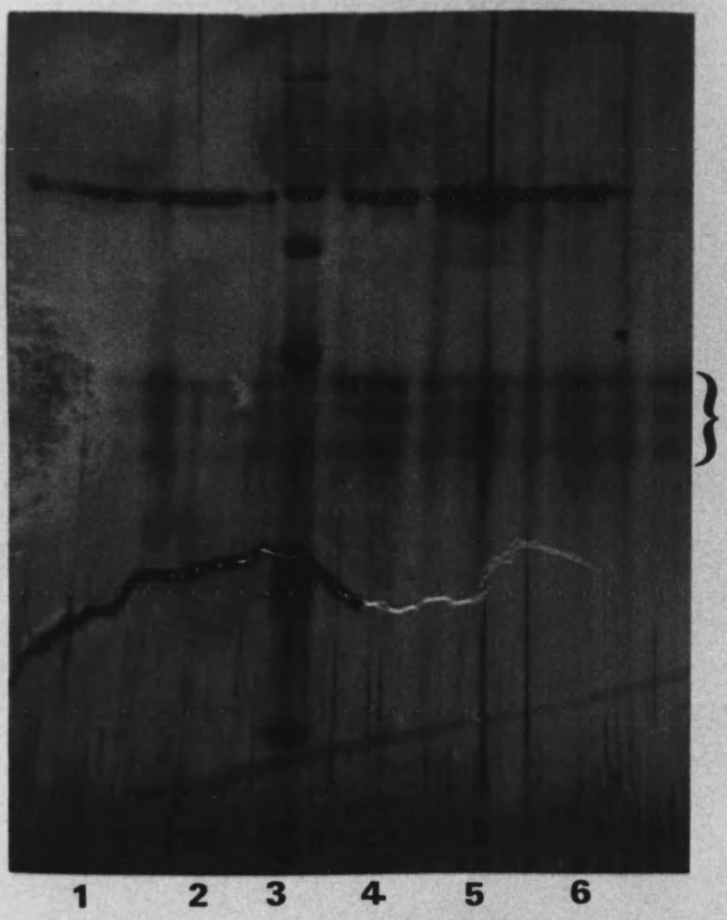


Plate 5 SDS-PAGE analysis of material electroeluted from 'Laemmli' preparative SDS-polyacrylamide gel (silver stained). Lanes 1, 2, 4, 5 and 6 represent fractions 1-5, and lane 3 standard high molecular weight markers, as described previously. The bracketed bands are artifactual and result from the use of β-ME as a reducing agent in the sample buffer.

500 µg affinity purified ADPRT was mixed with an identical volume of sample buffer (0.2 M sodium phosphate pH 7.2, 4% w/v SDS, 20% v/v glycerol, 10% v/v β-ME and 0.002% w/v Bromophenol blue) in a microfuge tube and placed in a 100 °C water bath for 3 minutes. Electrophoresis was carried out at 25 mA until the dye front had migrated to the bottom of the gel. The reservoir buffer comprised SDS-phosphate buffer diluted 1:1 with distilled water.

The 116 Kd component was clearly visible after electrophoresis and was excised and electroeluted as described previously, using the appropriate buffers. Fourteen fractions of 1 ml were taken and assayed for protein by a modified version of the standard Lowry assay (Markwell *et al.*, 1981) (Figure 35).

SDS-PAGE analysis of the fractions was not carried out, as the overall recovery of protein was so low (21%). The most concentrated fraction was number 5, with an estimated protein content of 14 µg/ml. The total recovery was still in sub-nanogram levels and therefore unsuitable for NH_2-terminal analysis.

The estimated recovery observed previously, using passive elution (section 5.3.1) was not considered to be significantly lower than this and accordingly this electrophoresis system was not employed in further studies.

5.3.4 Preparative SDS-PAGE using a Tris.borate buffer system

A final attempt at electroelution was carried out using the following SDS-Tris.borate system. A 7.5% w/v resolving gel was used with no stacking gel.

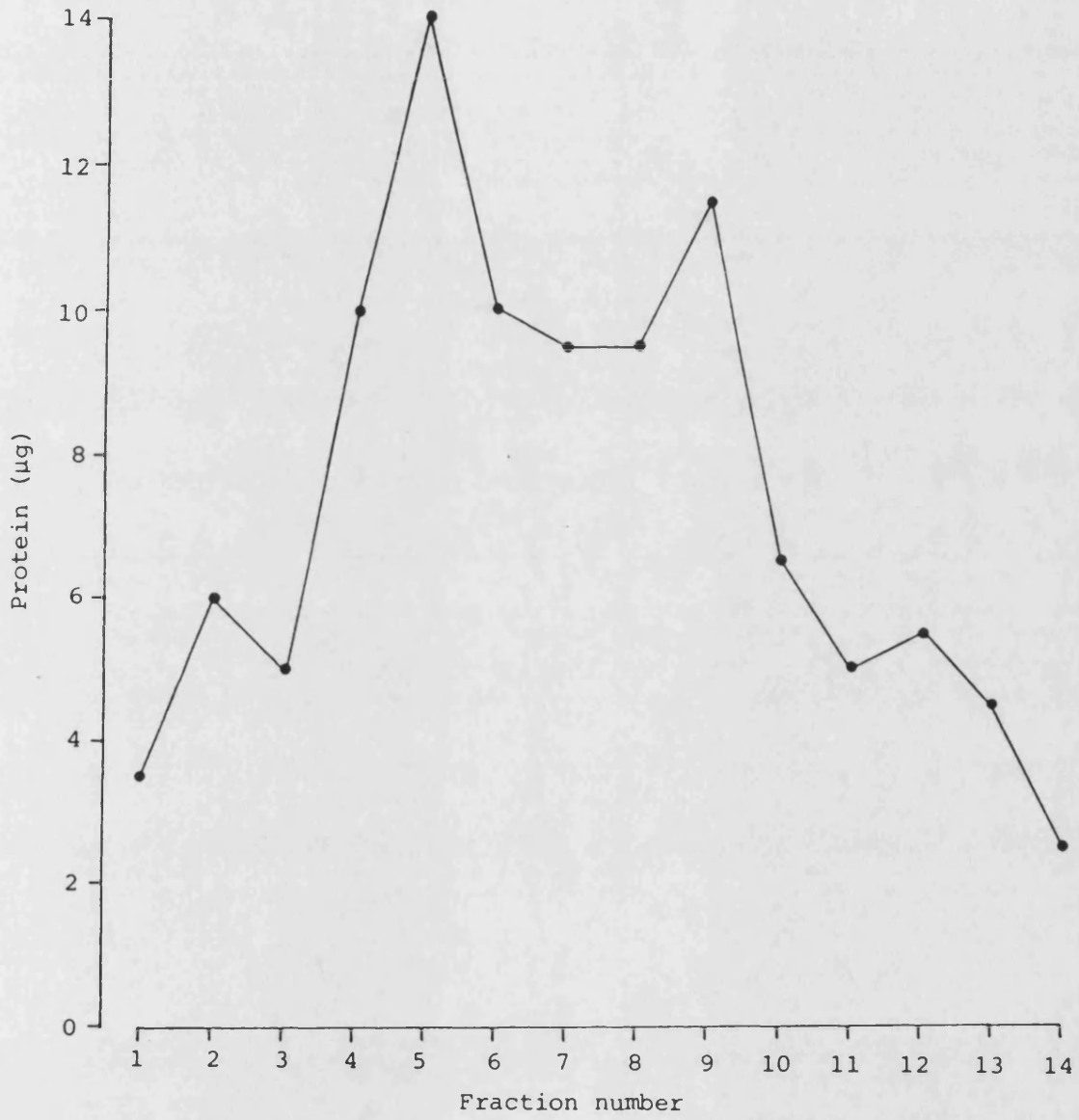


Figure 35 Electroelution of ADPRT.

500 µg affinity purified ADPRT was resolved by SDS-PAGE using the buffer system of Weber and Osborn (1969). Following excision of the ADPRT containing gel, elution was carried out as described in the text.

12.5 ml stock acrylamide (% T = 30, % C = 2.67), 26 ml distilled H_2O, and 20 µl TEMED were mixed well and degassed. 11.4 ml 1.5 M Tris.HCl, 0.4% w/v SDS, pH 8.8 and 60 µl 10% w/v ammonium persulphate were added, mixed quickly and the gel cast.

500 µg of sample was prepared as previously in an equal volume of 125 mM Tris.HCl pH 8.3, 4% w/v SDS, 20% v/v glycerol, 10% v/v β-ME, 0.002% w/v Bromophenol blue. Electrophoresis was carried out with electrode buffer of 25 mM Tris, 40 mM H_3BO_3, 1% w/v SDS pH 8.3, at a constant 35 mA, until the tracking dye had migrated to the bottom of the gel.

Immediately post-electrophoresis, the gel was processed as previously and fractions collected. The electroeluted fractions were dried down and redissolved in 100 µl distilled H_2O. 20 µl of each fraction was then analysed by SDS-PAGE in the Laemmli system followed by silver staining (Plate 6A).

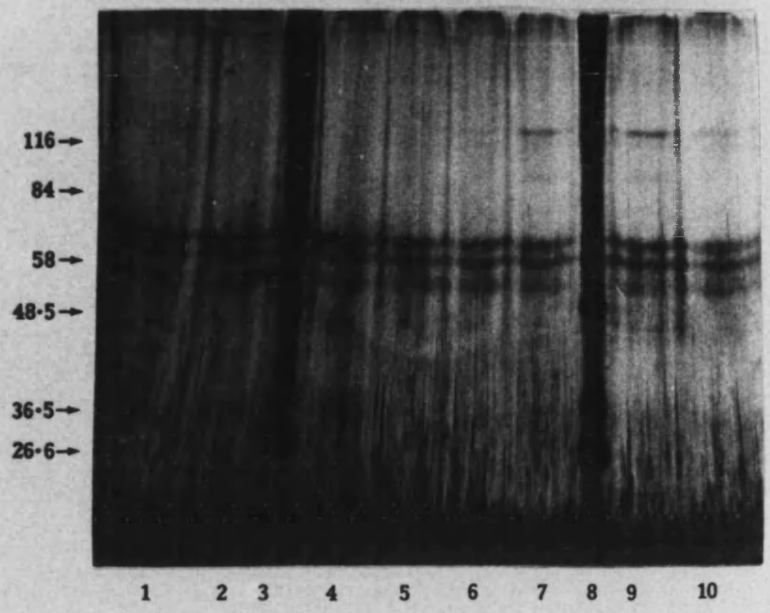
Significant amounts of protein were visible only in fractions 6 and 7, although in amounts clearly inadequate for further analysis. To ensure that protein was being eluted from the gel, the eluted gel piece was removed from the rod and stained with Coomassie Blue as previously (Plate 6B).

The speckled appearance of the gel was indicative of unsuccessful elution. As the preparative system was not cooled during the experiment, the probable cause of this was denaturation due to heat generation.

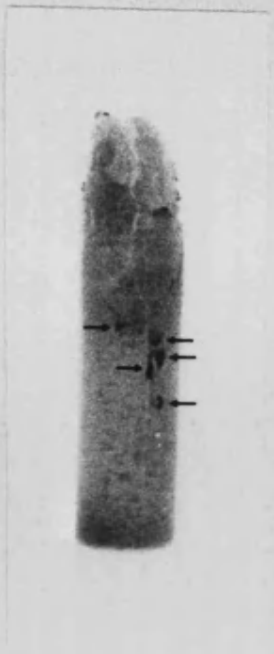
PLATE 6 (a) SDS-PAGE analysis of material electroeluted from Tris.borate preparative SDS-polyacrylamide gel (silver stained). Lanes 1, 2, 4, 5, 6, 7, 9 and 10 represent fractions 1-8. Lanes 3 and 8 represent prestained molecular weight markers (Sigma): β-Galactosidase (116 Kd), fructose-6-phosphate kinase (84 Kd), pyruvate kinase (58 Kd), fumarase (48.5 Kd), lactic dehydrogenase (36.5 Kd), triose phosphate isomerase (26.6 Kd).

(b) Coomassie Blue stained rod-gel, post-electroelution, showing non-eluted, denatured protein (arrowed).

Ⓐ


116
84
58
48·5
36·5
26·6
1 2 3 4 5 6 7 8 9 10

Ⓑ



5.3.5 Passive elution of protein from Tris.borate polyacrylamide gel

The failure of electroelution to obtain sufficient material for NH_2-terminal amino acid analysis prompted the use of passive diffusion as the recovery procedure after electrophoresis.

500 µg affinity purified material was separated in the Tris.borate system, as this was observed to give better separation than the phosphate system. Immediately post-electrophoresis, the gel piece containing the 116 Kd constituent was excised, divided into small pieces and added to a sterile tube containing 3 ml distilled H_2O. The tube was capped and agitated at room temperature for 20 h on a shaking water bath. The eluate was then removed and concentrated by ultrafiltration. The extraction was repeated twice more and the concentrated samples pooled.

Due to the difficulties encountered in assay for protein, the quantity recovered was estimated by visual comparison of stain intensity when 10 µl of the concentrate was run in the Laemmli SDS-PAGE system alongside 1, 2, 4, 8 and 16 µg of affinity purified material (estimated by dye binding assay). The stained band resulting appeared to correlate closest with the amount of protein seen in the 8 µg track (Plate 7). As the remaining total volume was 150 µl, this represented roughly 120 µg of polypeptide, fortunately the equivalent of 1 nmole, just sufficient for NH_2-terminal amino acid analysis.

Although visual comparison was not entirely satisfactory, linear densitometry could not be employed due to uneven distribution of protein in some of the bands (>2 µg).

PLATE 7 Quantitation of passively eluted enzyme.

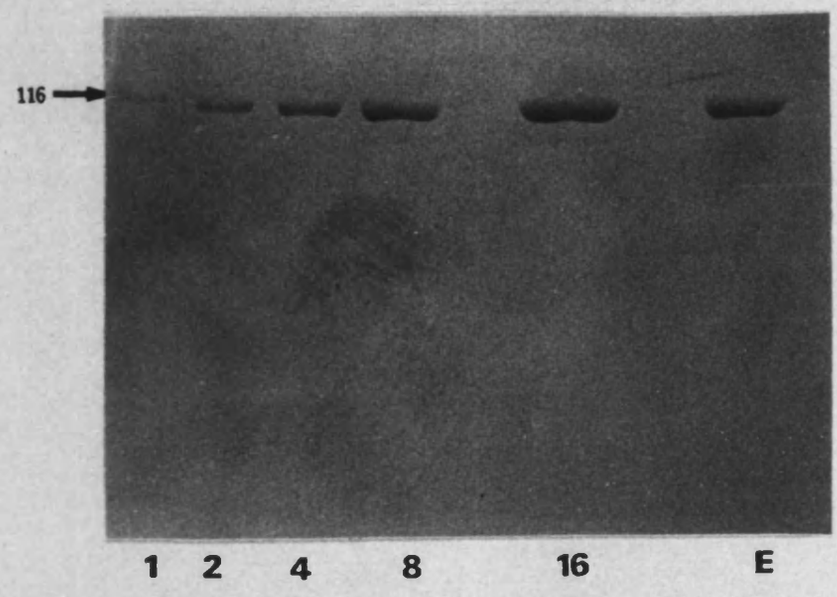


Plate 7 Quantitation of passively eluted enzyme. 1, 2, 4, 8 and 16 µg affinity purified ADPRT were analysed by SDS-PAGE alongside 10 µl (160 µl total) passively eluted enzyme.

5.4 NH_2-terminal Amino Acid Analysis

Covalent attachment of the NH_2-terminal amino acid of a polypeptide to a labelling substance followed by acid hydrolysis of the peptide-label adduct to its constituent amino acids may be employed to identify the NH_2-terminal amino acid of the peptide. To be successful, the covalent link must be stable to the hydrolysis conditions, and the derivatised amino acid must be recognisable after a subsequent separation step.

1-Dimethylaminonaphthalene-5-sulphonyl chloride (dansyl chloride) is frequently used as the label as it forms highly fluorescent sulphonamides when it reacts with free unprotonated primary amine groups. The linkage is stable to acid hydrolysis and the presence of only 1 nanomole of derivatised amino acid may be detected.

The method employed was that detailed by Walker (1984). The eluted material from the previous experiment was dried down in a 'dansyl' tube by vacuum dessication and processed as described.

Subsequent analysis of the TLC plate did not reveal a derivatised amino acid suggesting that the NH_2-terminus of the protein is modified, or blocked. Although the amount of material analysed was approaching the detection limits of the technique, this observation supported similar claims in the literature (section 1.3.1).

5.5 Cyanogen Bromide Cleavage of ADPRT

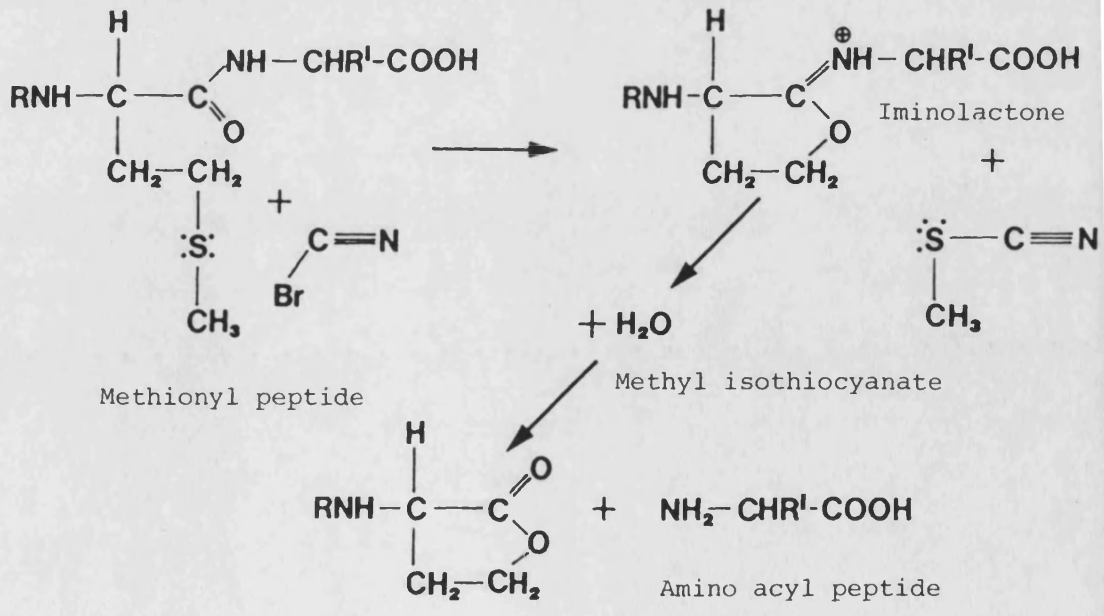
NH_2-terminal sequence studies could not be carried out on the purified protein *per se*, as sequence determination by the

Edman, Dansyl-Edman, and Dabsyl (4-N,N-Dimethylaminoazobenzene-4' isothiocyanate) methods require the amino group of the terminal residue to be freely available in an unprotonated form for coupling to take place.

Due to the unknown nature of the NH_2-terminal modification, generation of peptide fragments, with NH_2-terminal regions free for further analysis, was considered the best available option.

Cleavage of polypeptides can be carried out specifically by both enzymatic and chemical means. Trypsin digestion is an example of the former, which cleaves on the carboxyl side of lysine and arginine residues. An example of chemical cleavage is *via* hydroxylamine, which cleaves asparagine-lysine bonds. Cyanogen bromide, first used to cleave bovine pancreatic ribonuclease (Hirs *et al.*, 1960) was chosen for the fragmentation.

The reaction, given below, is carried out under mildly acidic conditions.



Cyanogen bromide was considered to be the ideal choice, as it reacts with methionine only, under conditions mild enough to avoid non-specific cleavage of other peptide bonds. Removal of excess reagent is also facilitated by its high volatility.

With methionine accounting for approximately 2% of the amino acid residues, 100% cleavage would generate 24 residues (calculated from an average amino acid mol. wt. of 100 d).

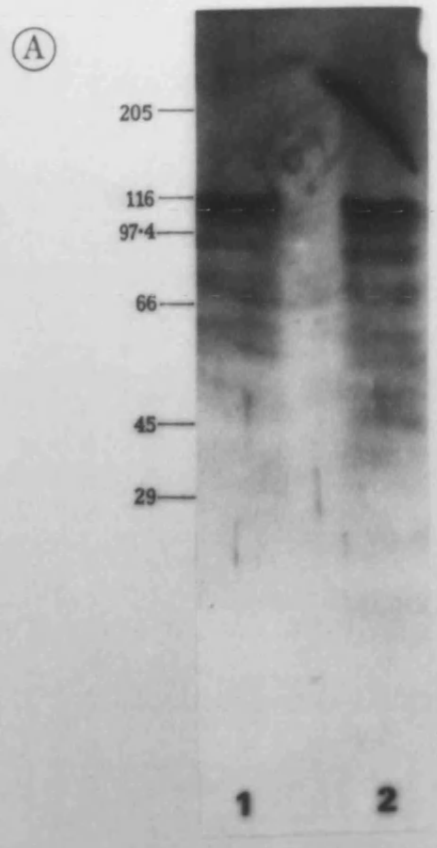
Cleavage reaction

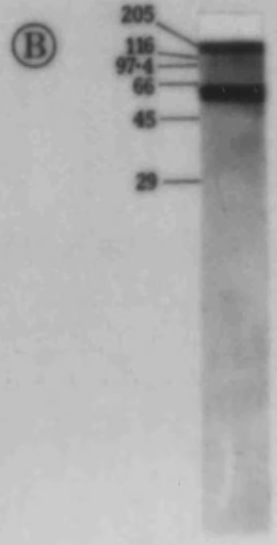
75% of the material in fraction 4 (section 5.3.2) was made up to 300 µl with distilled H_2O. 0.7 ml formic acid (Analar-BDH) was added to the tube and cyanogen bromide (10 mM in formic acid) added to the tube to give a minimum 100 fold excess mole for mole, assuming 100% total recovery *via* electroelution. The tube was vortex mixed, covered in foil and left at room temperature for 24 h. The solvent was then removed in a vacuum dessicator and the fragmented protein redissolved in 100 µl distilled H_2O. 5 µl of the resuspension was then analysed by SDS-PAGE on a linear 5-20% w/v gel, under non-reducing and reducing conditions, with silver staining (Plate 8A).

It was apparent that only partial cleavage had been achieved. Although a large number of smaller bands were visible, the major band at 116 Kd remained. The presence of β-ME at this stage had no noticeable effect. The remaining digest mixture was dried down in a vacuum dessicator and the reaction repeated for a further 36 h in 20% of the original volume.

PLATE 8 (a) SDS-PAGE (linear 5-20% w/v) analysis of cyanogen bromide digest of electroeluted material from section 5.3.2. Digested material was resolved under non-reducing conditions, lane A, and reducing conditions, lane B, *i.e.*, in the absence and presence of β-ME, and silver stained.

(b) SDS-PAGE (20% w/v) analysis of the remaining material, under reducing conditions, following digestion for a further period of 36 hours.


A
205
116
97·4
66
45
29
1
2


B
205
116
97·4
66
45
29

Further SDS-PAGE analysis (20% w/v to enable determination of molecular weights) under reducing conditions revealed cleavage of most of the material to peptides of approximately 50-60 Kd (Plate 8B).

Although not representing 100% modification of methionine residues, attempts to purify the resulting fragments by reverse phase high performance liquid chromatography (HPLC), were carried out. The object of the cleavage had been first and foremost to generate fragments with free amino termini. This had probably been achieved and it therefore seemed logical to attempt isolation of pure fragments.

5.5.1 Reverse phase HPLC

This is a rapid, highly reproducible, sensitive technique used routinely for the separation of peptides on the basis of differences of hydrophobicity. Usually the chromatographic adsorbent is silica based, derivatised with C4, C8 or C18 aliphatic arms, depending on the nature of the peptides. Once bound, the peptides are eluted with increasing concentrations of organic solvents.

Optical density measurements at 220 nm (for all peptides) and 280 nm (for peptides containing Tyr and Trp) are sufficient for detection at a nanomolar level, while fluorescence measurement of Tyr and Trp residues and counting of radiolabelled peptides may be used to extend the sensitivity to picomole and subfemtomole levels respectively, although losses can be restrictive at such low levels.

Separation of the cyanogen bromide digest was attempted on a C4 column in a system actually set up to separate a mixture of synthetic 'octapeptides'.

The digest was freeze dried and resuspended in 100 µl 50% v/v acetonitrile. The solution was centrifuged and separation of 20 µl of the resuspension was attempted using the following gradient at a flow rate of 1 ml per minute. Solvent A was 0.1% w/v TFA in H_2O, solvent B was 0.1% w/v TFA in acetonitrile (HPLC grade-BDH).

Time (minutes)	% v/v B in A
0	5.0
0.3	5.0
27.8	60.0
30.0	99.9
32.0	99.9
34.0	5.0
45.0	5.0

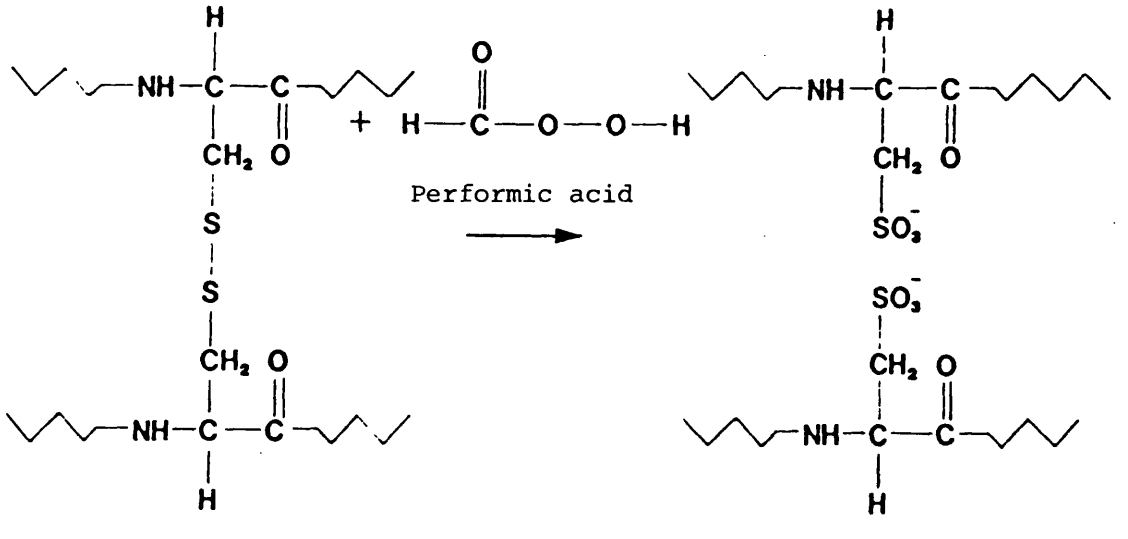
Elution was observed by measurement of the optical density at 220 nM. No peptide peaks were observed. This was probably due to non-interaction of the digested material with the adsorbant and emergence of the peptides immediately post-void volume. This may have resulted from the hydrophobic domains of the large peptides being inaccessible to the column. Often hydrophobicity of a protein is confined largely to the interior of the molecule with charged hydrophilic regions occupying the surface. The length of the aliphatic arm may also have been too short for significant hydrophobic interaction and therefore retention of fragments to occur (C8 and C18 columns appear to be preferred for such separations).

Another possibility is that the SDS-PAGE banding pattern observed did not reflect the state of the digest as it was applied to the column, although cleavage at methionine residues appeared partly successful when analysed in the presence of β-ME. Disulphide bridges remaining in the absence of β-ME could have been responsible for holding the fragments together and effectively masking the fragment generation. If lack of interaction was not responsible, it seemed likely that irreversible binding to the guard column, included to protect the separating column, may have been to blame.

Finally, and probably the most unlikely possibility, was that the amount of material loaded was insufficient for detection.

5.5.2 Performic acid oxidation (Weber *et al.*, 1972)

Irreversible modification of any cystine residues was carried out to eliminate the possibility of inter-fragment cross-linking. This may be done by carboxymethylation, but in this case was carried out by performic acid oxidation, resulting in the formation of cysteic acid residues.



Cystine

Cysteic acid

0.1 ml 30% v/v H_2O_2 was mixed with 0.9 ml 88% v/v formic acid and the mixture allowed to stand for 2 h at 25 °C. Half of the remaining digest was dried and dissolved in 100 µl of this reagent and left for 1 h at 0 °C. 1 ml distilled H_2O was added and the mixture freeze dried. The precipitate was dissolved in 50 µl 50% v/v acetonitrile and separation attempted as previously. Again, no peaks were observed.

CHAPTER 6

Immunochemical Studies

6.1 Use of Antibodies as Biological Tools

Exposure of vertebrates to any molecule or substance recognisable as foreign, results in production of proteins, termed immunoglobulins or antibodies, which are capable of interacting specifically with the molecule or substance. A substance which elicits such a response is termed an antigen and is recognised immunologically by distinct conformational determinants, or epitopes on it surface (Atassi, 1977).

As an antigen may comprise just six amino acids or six saccharide units (Kabat, 1968), the great potential diversity of antibody species is easily appreciated. The highly specific nature of antibody/antigen interactions therefore represents a biological means of generating highly specific probes for the detection and isolation of a whole host of cells, macromolecules and small organic molecules.

Only the methodology utilised, and not the immune response *per se* is discussed in any detail, as the primary requirement was ADPRT specific antiserum for further study of the enzyme.

6.2 Methodology

6.2.1 Immunisation of animals

When antibody is required as a specific probe, as in this case, it is desirable that the exposure step, or immunisation, should involve antigen in as pure a form as possible.

The use of affinity purified ADPRT as immunogen could not be considered, therefore, as the nature and relative antigenicities of the contaminating species had not been determined.

Although excellent separation had been observed by SDS-PAGE, protein recovery had proved inconsistent and difficult to monitor (Chapter 5). Accordingly, the method of Sakakibara and co-workers (1987) was employed, which circumvents the problem of antigen purity by use of SDS-agarose electrophoresis, followed by immunisation with the separated antigen/agarose gel piece.

SDS-agarose gel electrophoresis

A 5% w/v agarose gel (Sigma - Type VII) in 25 mM Tris, 190 mM glycine, 0.1% w/v SDS (electrophoresis buffer) was poured in a Pharmacia submarine minigel electrophoresis apparatus (gel size 10 x 7 x 0.4 cm). Two sample wells were formed, 10 x 1 mm and 60 x 1 mm, by insertion of a comb, and after setting, the gel immersed in electrophoresis buffer.

What was the source of ADPRT for SDS-Agarose gels

The sample 'in a suitable volume' was prepared identically as described and electrophoresis carried out at 50 V alongside high molecular weight SDS-markers, until the tracking dye had reached the end of the gel. The slab was then quickly removed and protein bands visualised by brief staining in Coomassie Blue (0.1% w/v in 10% v/v acetic acid, 10% v/v methanol) followed by destaining in the same solvent. The gel containing the required antigen was excised with a clean scalpel and processed for immunisation as described below.

Immunisation procedure

The gel piece containing antigen was placed in a clean glass syringe and 2 volumes of PBS added. The syringe was then placed in a 65 °C water bath and the agarose allowed to melt, followed by gentle inversion to mix the contents. The syringe was then connected *via* a stainless steel tube (internal diameter 1 mm) to an identical syringe containing 2 volumes of Freunds Complete Adjuvant (Sigma) (Incomplete when boosting animals). A water in oil emulsion was then prepared by injecting the aqueous phase into the adjuvant, followed by repeated forcing of the mixture from one syringe to the other until a smooth, creamy consistency was obtained.

Injection of the antigen was then carried out either intramuscularly (i.m.) at two sites (1 in each hind leg of the rabbit), or subcutaneously (s.c.) at four sites (along the back of the animal).

Immunisation protocol

Three female New Zealand white rabbits (3-3.5 kg) were immunised by i.m. injection of approximately 90 µg ADPRT and boosted with 50 µg ADPRT s.c. at intervals of 14 and 28 days post immunisation. Five days after the second boost, 10 ml of blood was collected from the ear vein of each animal and tested for the presence of anti-ADPRT antibodies.

Preparation of serum

The blood was collected in 15 ml Corex tubes and allowed to clot for 2 h at room temperature. The tubes were then ringed and

transferred to a fridge at 4 °C overnight for the clot to contract. The tubes were then centrifuged at 2500 g for 30 minutes at 4 °C and the serum frozen at 20 °C in 0.5 ml aliquots until required.

6.2.2 Precipitation reactions

Antibody/antigen interactions are frequently visualised by agglutination and precipitation reactions.

Agglutination is employed with insoluble antigen systems and is a highly sensitive means of antibody detection requiring relatively small amounts of antibody to effect cross-linking and subsequent visible clumping of antigen species.

Precipitation reactions are used for soluble antigen systems and occur when antibody and antigen interact under optimal conditions, *i.e.*, antigen must be present in high enough levels for visualisation of precipitate, but must not be present in over large excess, because soluble antibody/antigen complexes result.

Precipitation occurs in most systems involving a multivalent (multiepitope) antigen and bivalent antibody species and presents a highly versatile means of detection of antigen or antibody in solution, tissue, or cells.

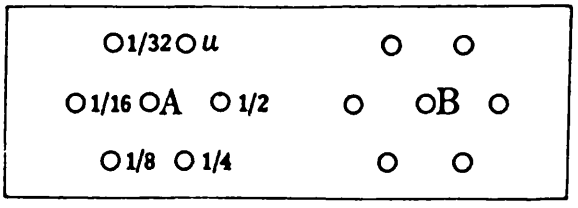
Precipitation reactions are usually carried out in agarose gels where convective currents, often observed in liquid systems, are avoided. The techniques are also easy to perform, offer high sensitivity, precision and resolution and permit analysis of a variety of different antibody and antigen preparations in the same system.

Double immunodiffusion studies

The system developed by Ouchterlony (1958) was used for initial studies. In this method, two separate wells are cut in an agarose gel, one containing the antigen solution and the other containing antiserum. Diffusion is allowed to take place into the gel towards the adjacent wells, forming concentration gradients of the two species. Where specific antibody reacts with antigen under the right concentration constraints, precipitation occurs which is visible as a pale white line or arc.

The technique has great qualitative value in determining relative heterogenicities of antibody and antigen preparations, but was used in this case only to determine the presence or absence of antibodies to ADPRT.

Microscope slides (75 x 25 mm) were precoated with 0.5% w/v agarose and dried thoroughly. 3.75 ml hot agarose (1% w/v in PBS) was carefully added to each slide and allowed to set at room temperature. Wells were then cut into the gel with a cork borer, in the pattern shown below, and the gel plugs removed by suction.



In centre well A was placed antigen solution (1 mg/ml) and in centre well B neat antiserum. Serial two fold dilutions of antibody in PBS were placed in the radial wells around centre well A and dilutions of antigen in the wells around centre well B. The

solutions were left to diffuse for 24 to 72 h at room temperature in a humid container and observed for precipitation arcs at regular periods. When precipition arcs were observed, they were stained, to enhance the contrast, with Coomassie Blue (section 2.4).

6.2.3 Immune inhibition of ADPRT activity

Presentation of a large polypeptide such as ADPRT to an animal offers a large variety of epitopes capable of evoking a response. To determine whether the immunisation procedure had successfully elicited production of antibodies to any of the domains of the protein critical for enzymatic activity, a number of immuno precipitation reactions were set up.

10, 1 and 0.1% v/v dilutions of heat inactivated sera (56 °C for 45 minutes) in 0.3 M salt extract of pig thymus nuclei were incubated overnight at 4 °C. 50 µl of the mixture was then transferred to a tube containing 450 µl reaction mixture, *i.e.*, 0.1 M TEA.HCl pH 8.2, 10 mM $MgCl_2$, 2 mM DTT, 500 nM NAD^+ (20 µCi/nmole) and 20 µg DNA, at 26 °C. Aliquots of 20 µl were taken over 3 minutes and measured for ADPR polymer by the acid washed disc method. Rates were calculated using linear regression analysis on all data.

6.2.4 The enzyme linked immunosorbant assay (ELISA)

Precipitation, like most serological techniques, is qualitative and accordingly, a means of semi quantitatively measuring the response was sought.

Although the use of radiolabelled antibodies and antigens (in particular) has gained great popularity for quantitative studies of

immunological interactions, there are unfortunately a number of drawbacks associated with their use. As well as being expensive to carry out, the techniques require hazardous reagents with short shelf lives. The use of non radioactive labelling therefore presents an attractive alternative.

In the mid 1960's, the coupling of an antibody to an enzyme label without loss of immunological and enzymic activity opened the door for widespread use of such labels. Initially such conjugates were used for microscopical detection and localisation of cellular antigens (Nakane *et al.*, 1966a,b). However, following the similar observation that antigens and antibodies could be immobilised on insoluble supports without loss of activity, the first enzyme linked immunosorbant assays (ELISA) were developed (Engvall and Perlmann, 1971; Van Weeman and Schuurs, 1971). These ELISA techniques directly measure antibody/antigen interactions and are comparable in sensitivity to RIAs (Engvall and Carlsson, 1976), while existing as cheaper, safer, more easily applicable alternatives.

Choice of assay

The indirect micro ELISA method, carried out in microtitre trays, was chosen.

Polystyrene was selected as the insoluble support as it has been shown to bind a large number of protein species effectively, including glycoproteins and lipoproteins, as well as glycolipids, denatured DNA and bacterial lipopolysaccharides (Engvall and Carlsson, 1976). Antigen is adsorbed onto the support and the

plate incubated with dilutions of the test serum. Excess unreacted serum is washed away and a second immunoglobulin, raised against total immunoglobulin from the test species, conjugated to the enzyme is added. After incubation, the plate is washed again and incubated with a relevant substrate until detectable product develops. The reaction is then stopped and the product quantified by a suitable means.

Choice of enzyme

The enzyme to be conjugated must be cheap, of high purity, stable and reactive, while the product of the reaction should be soluble and have a high molar extinction coefficient.

Alkaline phosphatase has been used extensively, although horse radish peroxidase (HRPO) is gaining increasing popularity due to the wide variety of available substrates and the ease with which conjugation can be carried out.

In all ELISA and Western blot experiments (described later) the enzyme used was HRPO conjugated to goat-anti rabbit IgG (Sigma).

Assay method

All incubations were carried out on a shaking water bath.

(1) Coating of adsorbant with antigen

Antigen in 100 µl coating buffer [50 mM Bicarbonate buffer pH 9.6 containing 0.01% w/v Thiomersal (BDH)] was added to each well and adsorption allowed to take place for 2 h at 37 °C or overnight at 4 °C.

(2) Washing

The excess antigen was removed from each well by means of a short form pasteur pipette attached to a suction pump, and the plate 'rapped' on a clean, paper covered bench to remove residual non reacting material. 200 µl of wash buffer was added (50 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.05% v/v Tween 20, 0.2% w/v Casein and 0.005% w/v Thiomersal) and after 10 minutes the buffer was removed as above. It is important to carry out this step efficiently, as the presence of non reacting material can significantly raise non specific background levels. To ensure this, the wash procedure was carried out three times at each stage.

(3) Incubation with test serum

Serial dilutions of the test sera in wash buffer were made and 100 µl aliquots added to each well. Incubation was carried out at room temperature for 2 h, followed by removal of unreacted antibody and washing of the plate.

Have we the time determined 1/1/4 2 hrs ?

(4) Incubation with conjugate

100 µl of conjugate, diluted 1 in 1000 with wash buffer supplemented with 5% v/v normal goat serum was added to each well and incubation for 2 h followed by washing carried out as above.

(5) Addition of substrate

Due to the instability of the substrate, fresh reagent had to be made up each time immediately prior to use:

0.1M acetate/citrate buffer pH 6.0, containing 0.05% v/v Tween 20 and 100 µl tetramethylbenzidine (TMB) solution

(10 mg/ml crystalline TMB in DMSO) was mixed with H_2O_2 (2 µl per 10 ml substrate solution) and if the solution remained clear for 2 minutes, used immediately.

100 µl was added to each well and incubated at room temperature until sufficient colour developed. The reaction was stopped by addition of 25 µl 2 M H_2SO_4, which turns the product from blue to yellow.

Substrate was quantified by reading the plate on a spectrophotometer at 450 nm.

In each case, a negative control comprising serial dilutions of normal (non-immune) rabbit serum was run.

6.2.5 Western blotting of proteins

Although SDS-PAGE is a very powerful biochemical tool, further study of separated species is often extremely difficult due to reasons of inaccessibility. Use of enzyme substrates to visualise species has been tried (Dulaney and Touster 1970), but is of limited application.

The recognition that most proteins bind underivatised nitrocellulose sheets in a non covalent manner (Kuno and Kihara, 1967) has made the medium an ideal choice in many solid phase binding assays. Southern (1975), recognising that DNA also binds nitrocellulose, devised the first means of transferring electrophoretically separated species to nitrocellulose. Similar blotting of protein gels followed (Bowen *et al.*, 1980; Renhart *et al.*, 1979). Passive transfer methods, however, are molecular weight and concentration dependant for efficient transfer, proving restrictive with some large protein species.

Electrophoretic transfer (Towbin *et al.*, 1979; Bittner *et al.*, 1980) is a far more efficient, quicker and easily controlled alternative. The high fidelity of transfer and the availability of the transferred species for further analysis has seen extensive adoption of the technique for study of complex antigen and antibody mixtures.

Although the detection of transferred species is primarily determined by the titre of the test serum, use of radiolabelled or enzyme linked secondary antibodies have allowed visualisation of picogram levels of some transferred species.

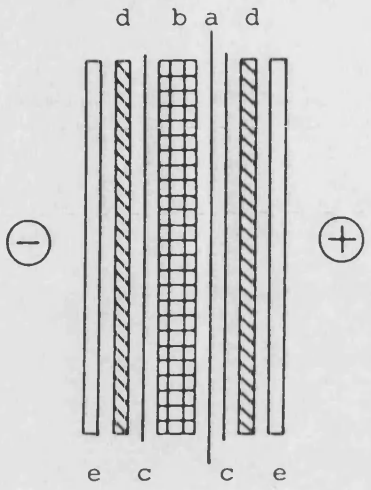
In terms of immunological recognition, there was concern that the transferred species might not have maintained their conformational integrity (Tsang *et al.*, 1983). The presence of SDS and the binding process itself are both possible means of profoundly altering the array of available antigenic determinants. Bowen and co-workers (1980), using the DNA binding capacity of nuclear HeLa proteins as an index of structural integrity, found that such fears appeared to be largely unfounded. Structural disruption can be minimised further, however, by passive or electroblotting of proteins from IEF gels (Reinhart and Malamud, 1982).

Western blotting protocol

(a) Protein transfer (Bittner *et al.*, 1980)

(When handling nitrocellulose, disposable gloves were always worn to reduce contamination.) Immediately post-electrophoresis, the stacking gel was removed and the resolving gel soaked in blotting buffer (25 mM sodium phosphate pH 6.5) for 15 minutes. 0.45 μm pore nitrocellulose (Sartorius) was thoroughly pre-wetted in blotting

buffer, taking care to avoid air bubbles, and the transfer cassette set up as shown.



a Nitrocellulose
b Gel
c Whatman 3 MM
d Sponges
e Cassette

Ensuring that the nitrocellulose was on the anodal side of the gel, the assembled cassette was placed in a Transblot (BioRad) electro-blotting tank filled with blotting buffer and agitated briefly to remove any air bubbles. The electrodes were connected and transfer carried out overnight at 10-15 V.

(b) Probing with test antibodies

After blotting, the nitrocellulose was transferred to a shallow tray and washed four times for 10 minutes with PBS-Tween 20 at room temperature with gentle agitation. (Agitation was used throughout to ensure uniform interaction of reagents.) Visual-isation of the transferred protein was then achieved by immersion of the nitrocellulose in Ponceau S solution (0.1% w/v in 3% w/v TCA) for 0.5 to 3 minutes, followed by partial destaining with successive changes of distilled H_2O. When appropriate, lanes were dissected with a clean scalpel and transferred to vessels suitable for further treatment; *i.e.*, either permanent staining or antibody

treatment, followed by complete destaining with distilled H_2O then PBS-Tween 20.

The enzyme linked conjugate described previously was used to visualise antibody/antigen interactions.

The sites on the nitrocellulose not occupied by transferred protein were then blocked by incubation with wash buffer (PBS-Tween 20 containing 1% w/v Casein) at 37 °C for 3 h, or overnight at 4 °C. The wells were drained and aliquots of test sera, diluted in wash buffer, were added and incubated at room temperature for 3 h. The strips were then rinsed three times with wash buffer, 10 minutes each, and conjugate added, diluted 1 in 1000 with wash buffer containing 2.5% v/v normal goat serum. After 2 h at room temperature, the strips were washed again as above, followed by addition of substrate solution.

The substrate solution was made up fresh each time and consisted of 0.02% w/v aminoethyl carbazole (from a 0.4% w/v stock solution in DMF), 0.03% v/v H_2O_2 in 50 mM sodium acetate buffer pH 5.0. Incubation was carried out at room temperature until sufficient red reaction product was visible. The strips were then drained and rinsed in distilled H_2O, followed by drying and recording of results by photography.

Visualisation of protein transferred

(a) Amidoblack

A solution of 0.1% w/v amidoblack (Napthol Blue Black-Sigma) in 25% v/v isopropanol, 10% v/v acetic acid, was added to the strips and incubated for 5 minutes at room temperature. The strips were

then drained and destained in the solvent only, followed by rinsing with distilled H_2O.

Although of suitable sensitivity for most applications, this method has one inherent drawback in that the nitrocellulose shrinks slightly following this treatment. Comparative staining was therefore carried out in most cases using Indian ink.

(b) Indian ink staining (Hancock and Tsang, 1983)

The strips were washed extensively in PBS/Tween to remove interfering SDS and incubated in 0.7 µl/ml Indian ink in PBS/Tween. The strips were agitated at room temperature for 30 minutes, followed by rinsing with distilled H_2O.

6.3 Results

6.3.1 Ouchterlony double diffusion

The initial diffusion reaction was set up using salt extract of pig thymus nuclei (1 mg/ml) as antigen and serial antiserum dilutions down to 1 in 32. At room temperature, no precipitation arcs were visible after 72 h. Plate 9 shows the result after 36 h of carrying out an identical experiment with affinity purified ADPRT (1 mg/ml) as antigen. Precipitation arcs are clearly visible with animals 81 and 82 down to a serum dilution of 1 in 8, although no response was apparent with animal 107. Control serum showed no precipitation.

Although the result looked disappointing in terms of serum reactivity and antigen concentration, it was not entirely surprising when the means of presentation of antigen to the animal was considered. Epitopes on antigens exist in two forms, continuous and discontinuous. Continous epitopes are those determined

PLATE 9 Ouchterlony double diffusion

by a linear sequence of residues (amino acids in this case), whereas discontinuous epitopes exist by virtue of conformational folds in the molecule. The use of ADPRT as an immunogen after treatment with SDS would present the molecule to the animal with an artificially high level of continuous epitopes relative to the molecule in its native state. In the test described, only epitopes present on the outside surface of the molecule would be available for interaction with the antibody population and therefore interactions involving the whole antibody pool would not be observed. Such a phenomenon underlines the necessity of presenting the antigen in a manner appropriate to the desired response.

6.3.2 Immune inhibition of ADPRT activity (Figure 36)

Salt extract of pig thymus nuclei alone incubated after the overnight period gave an incorporation rate of 44 dpm per minute. Addition of normal, non-immune rabbit serum to levels of 1 and 10% resulted in an increase of the activity rate by a factor of 5 to 6, although this was not observed at a concentration of 0.1%. Incubation of non-immune serum alone with DNA, DTT, and radioactive NAD^+ resulted in no TCA precipitable product, however, indicating that the serum itself was not inherently responsible. As only bisulphite, a non-specific protease inhibitor was included in the nuclear extract, residual proteolytic activity throughout the overnight incubation period was the most likely cause of the reduced activity observed. The increased activities observed with elevated serum levels therefore probably reflect dilution of ADPRT proteolysis by addition of serum proteins.

The 0.1% v/v serum incubations were therefore not considered to reflect true inhibition of ADPRT by specific interaction with

Figure 36 Immune inhibition of ADPRT activity

10, 1 and 0.1% v/v dilutions of heat inactivated sera were incubated with nuclear extract for 18 h at 4 °C.

50 µl of each incubation mixture was then assayed for enzyme activity by the acid washed disc method.

The rates of formation of TCA insoluble product, calculated by linear regression analysis, are given, followed by the respective correlation coefficients in parentheses.

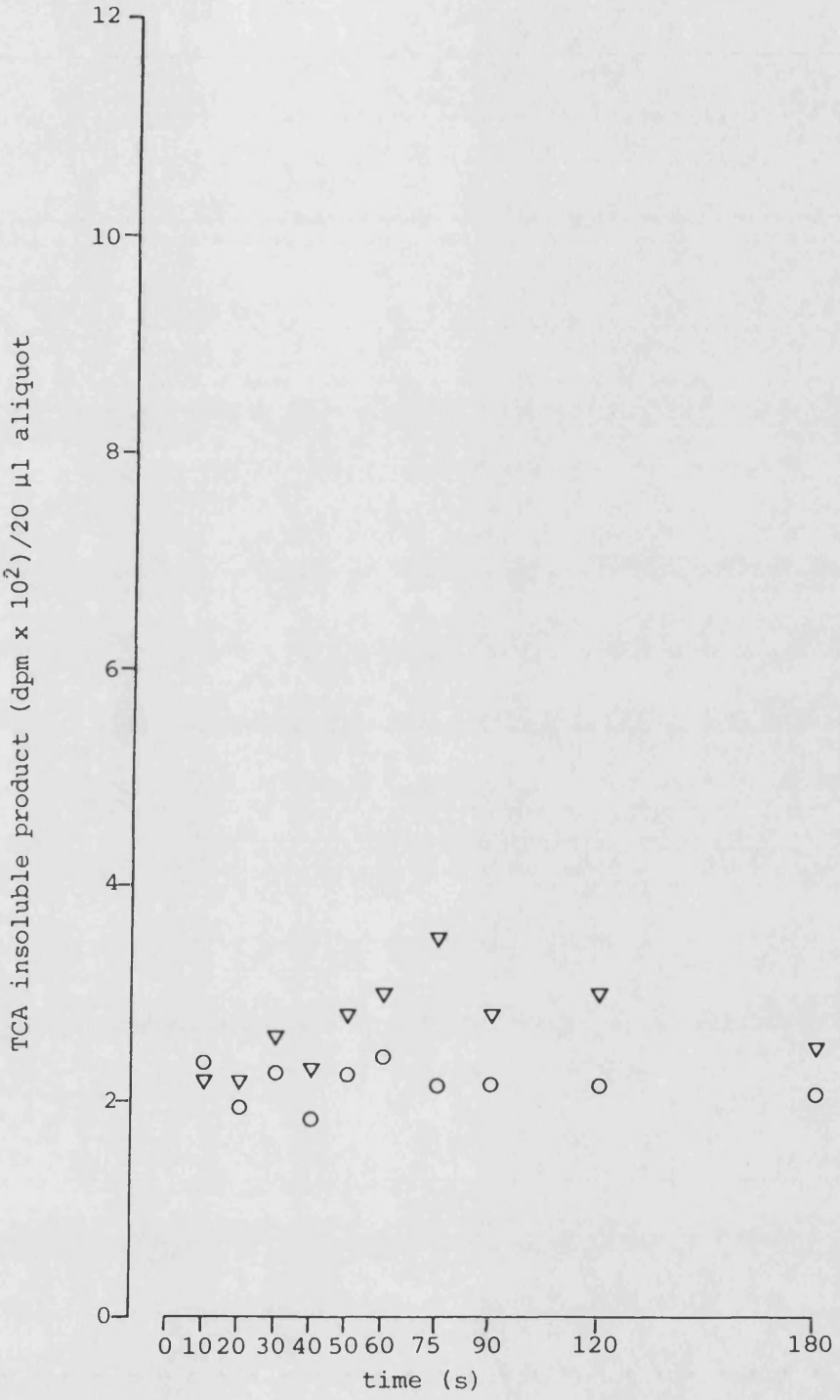


Figure 36a Nuclear extract control (▽): 44.2 (0.84)
Normal rabbit serum control (○): -2.6 (0.13)

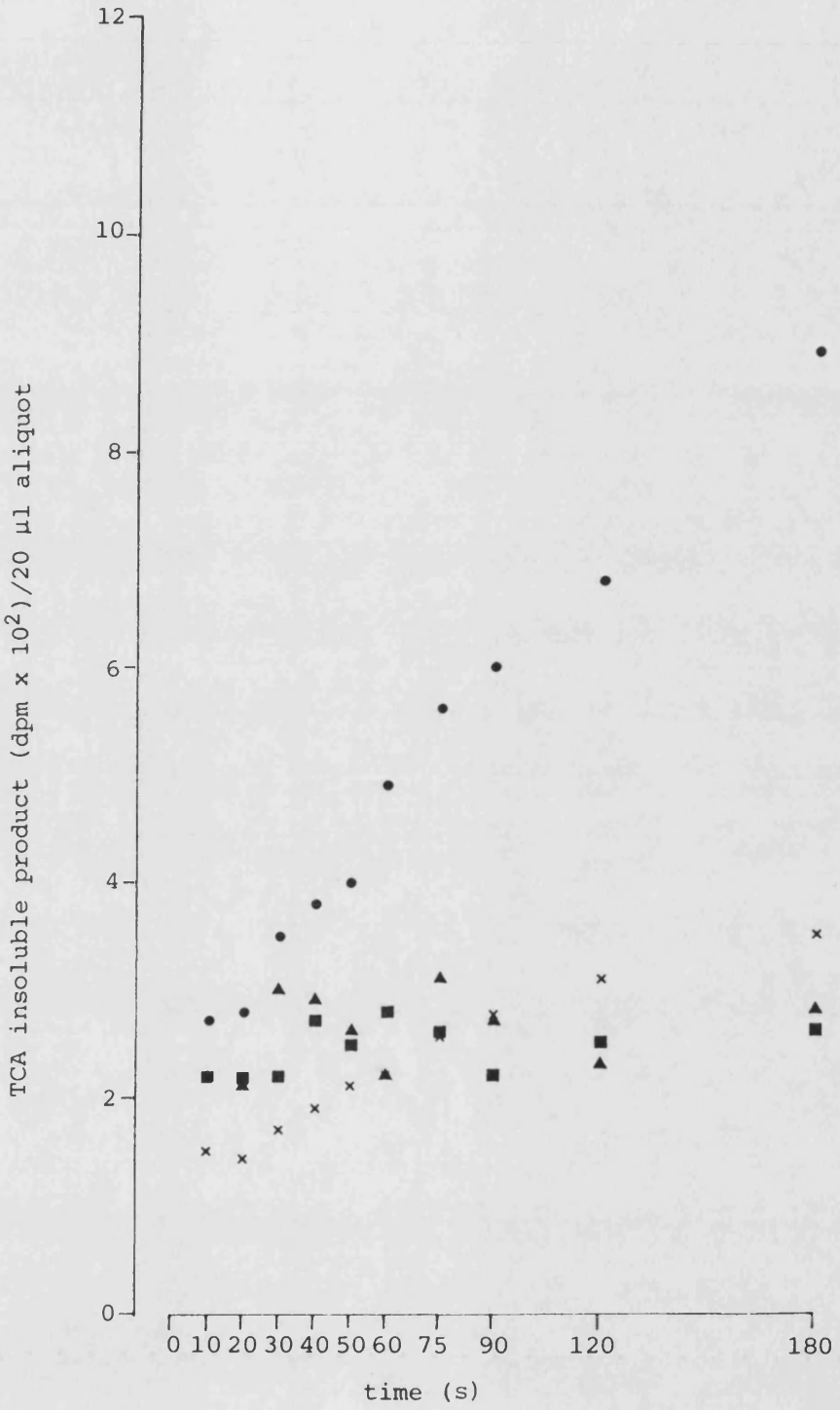


Figure 36b 10% v/v serum dilutions

Normal rabbit serum (●): 225.6 (0.99)
Serum 81 (▲): 9.8 (0.23)
Serum 82 (■): 10.0 (0.37)
Serum 107(×): 78.0 (0.98)

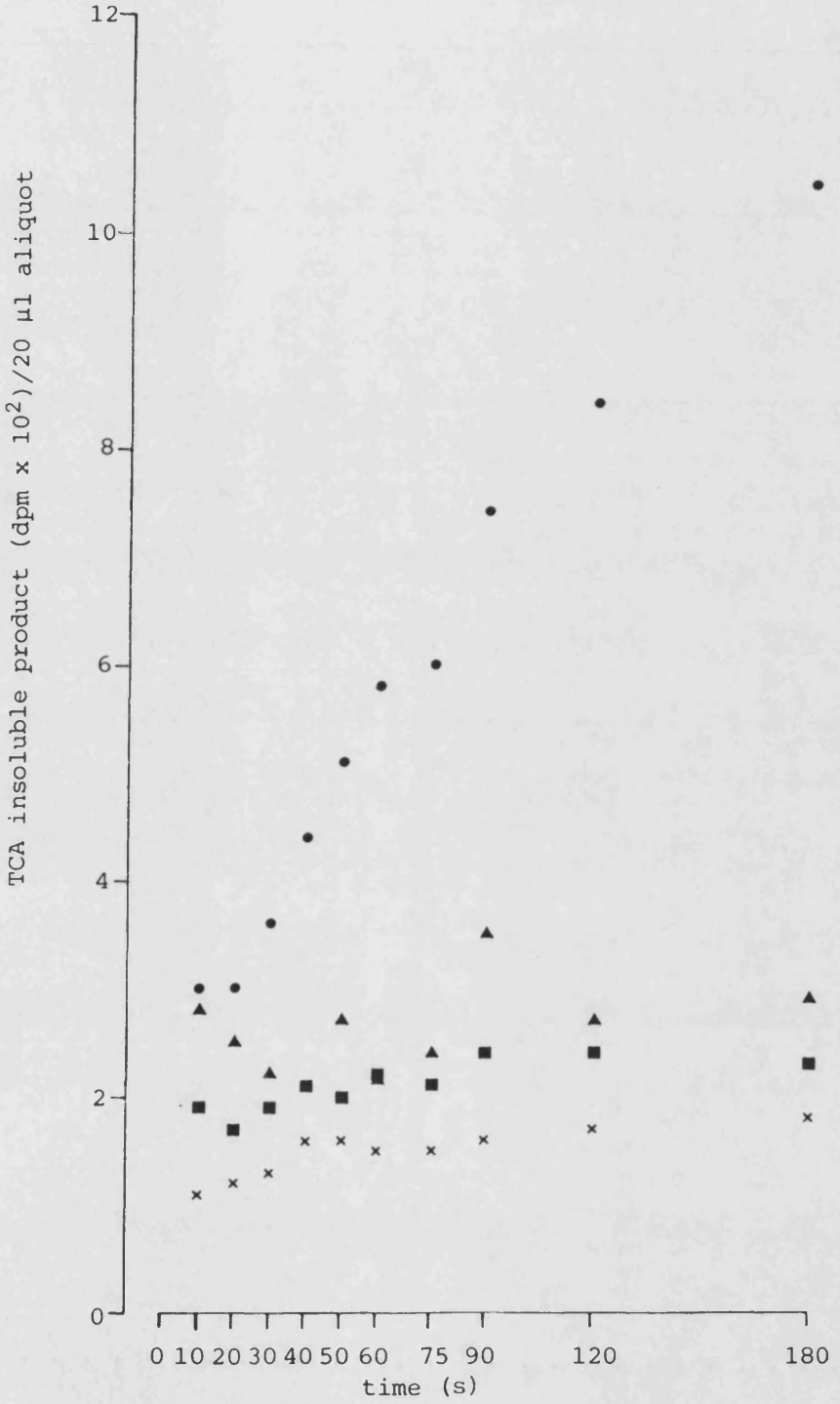


Figure 36c 1% v/v serum dilutions

Normal rabbit serum (●): 278.4 (0.99)
Serum 81 (▲): 17.2 (0.37)
Serum 82 (■): 21.0 (0.78)
Serum 107(×): 21.6 (0.83)

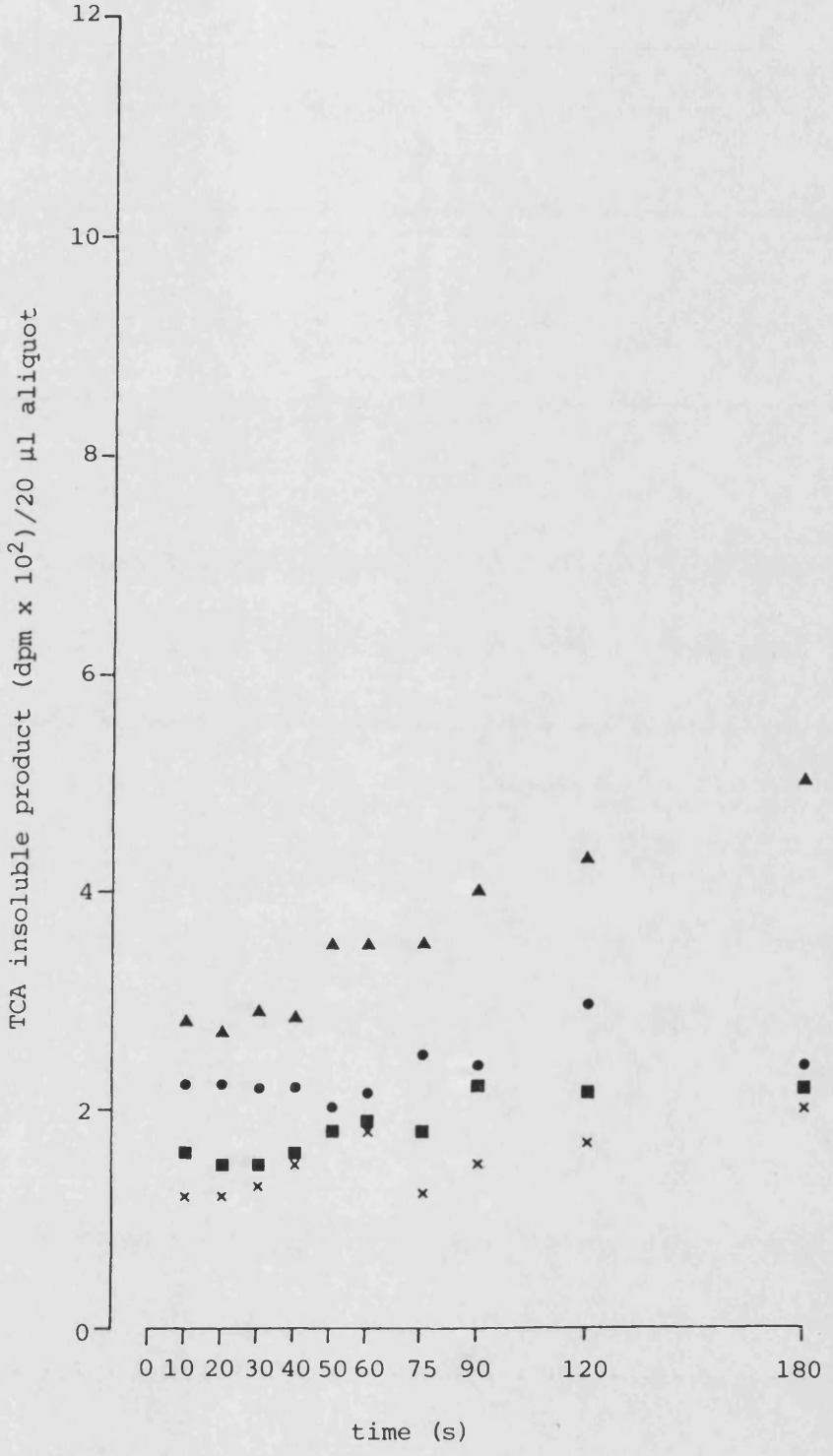


Figure 36d 0.1% v/v serum dilutions

Normal rabbit serum (●): 17.6 (0.57)
Serum 81 (▲): 85.0 (0.98)
Serum 82 (■): 28.4 (0.88)
Serum 107(×): 26.0 (0.83)

antibodies. The inhibition levels observed with the test sera at the higher dilution, however, strongly suggested specific antibody/antigen interaction.

At a 10% v/v dilution, serum 107 showed the weakest inhibition relative to the non-immune control, at 65%, with sera 81 and 82, exhibiting 96% inhibition. At 1% serum dilution, the inhibitions were 94, 92 and 92 respectively for sera 81, 82 and 107.

Although a somewhat cursory study, the inhibition observed represents very strong evidence for the presence of anti ADPRT antibodies in all three sera.

6.3.3 ELISA

When setting up an ELISA system, the concentrations of antigen and antibody which give maximal contrast between positive and negative test samples, while retaining low negative values, are usually determined by the chequerboard titration method. In order to quantitate the system accurately, standard preparations of antigen and antibody are therefore required. As this was not possible, due to the unavailability of a positive reference serum, only titre values relative to those of non immune serum could be determined.

Excess antigen (0.5 µg affinity purified ADPRT) was used in the immobilisation step and initial studies carried out with serial twofold dilutions of test sera from 1 in 100 to 1 in 204800. Normal rabbit serum (NRS) was used as the negative control because pre-immune serum was extensively contaminated by haemolysis and exhibited very high background values under the conditions described.

Figure 37A shows the end point titration values of the three test sera. Serum 82 showed the best response with a value of 1 in 102400, followed by serum 81 with 1 in 25600 and serum 107 with 1 in 6400. Although end point titres (the serum dilution at which the test curve joins the negative serum curve) are somewhat arbitrary, they sufficed as a convenient means of semi-quantitation.

Boosting of animals

To increase the titre of the three sera, particularly serum 107, the animals were boosted again 28 days after the previous boost with similar quantities of antigen. Five days post boost, the animals were test bled (*via* the ear) and analysed for reactivity by ELISA identically as before (Figure 37B). Although not directly comparable, as insufficient enzyme was available to carry out a parallel study with the previous serum, the end point titres were raised to 1 in 204800 for sera 81 and 82 and 1 in 102400 for serum 107. The animals were exsanguinated and their sera stored at -80 °C until required.

6.3.4 Characterisation of antiserum by Western blotting

Choice of serum dilution

The equivalent of 50 µg salt extracted nuclear protein was separated by 7.5% w/v SDS-PAGE using the Laemmli buffer system, transferred to nitrocellulose and probed with varying dilutions of antiserum 82.

Plate 10 shows that one major band was observed at all concentrations corresponding to approximately 116 Kd in size. A number of smaller bands were also visible, but very faint in comparison. The normal rabbit serum control showed no visible evidence of reactivity.

Figure 37 ELISA

Analysis was carried out as described in section 6.2.4 (0.5 µg affinity purified ADPRT immobilised per well).

(A) Result of assays on sera prepared from the immunisation protocol described in section 6.2.1.

(B) Result of assays on sera prepared from animals following a further booster injection.

(28 days after the second boost, another injection was administered and sera prepared 5 days later.)

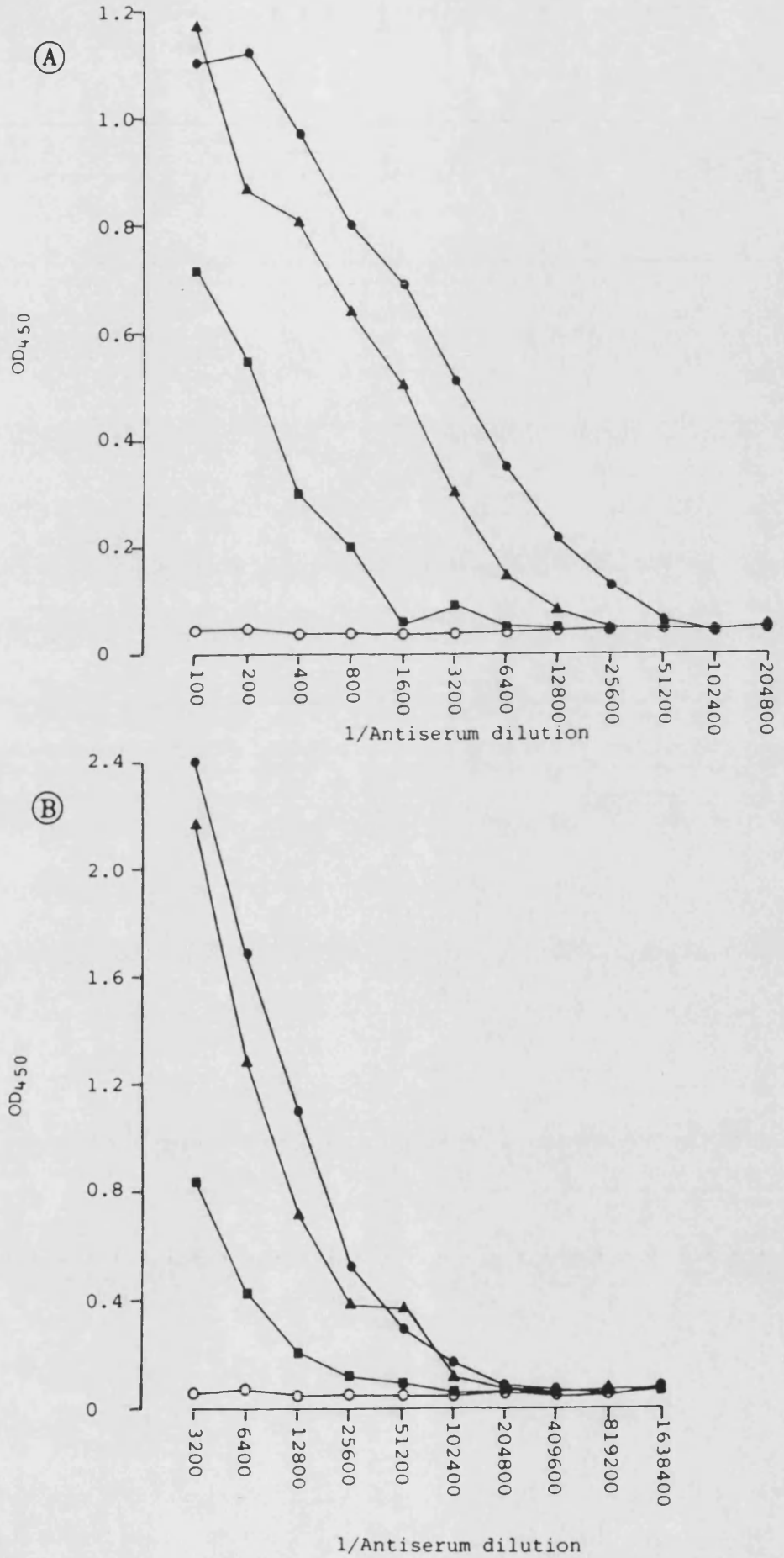

A
1.2
1.0
0.8
0.6
0.4
0.2
0
OD450
100
200
400
800
1600
3200
6400
12800
25600
51200
102400
204800
1/Antiserum dilution
B
2.4
2.0
1.6
1.2
0.8
0.4
0
OD450
3200
6400
12800
25600
51200
102400
204800
409600
819200
1638400
1/Antiserum dilution

PLATE 10 Characterisation of antiserum by Western blotting:

(1) Choice of serum dilution

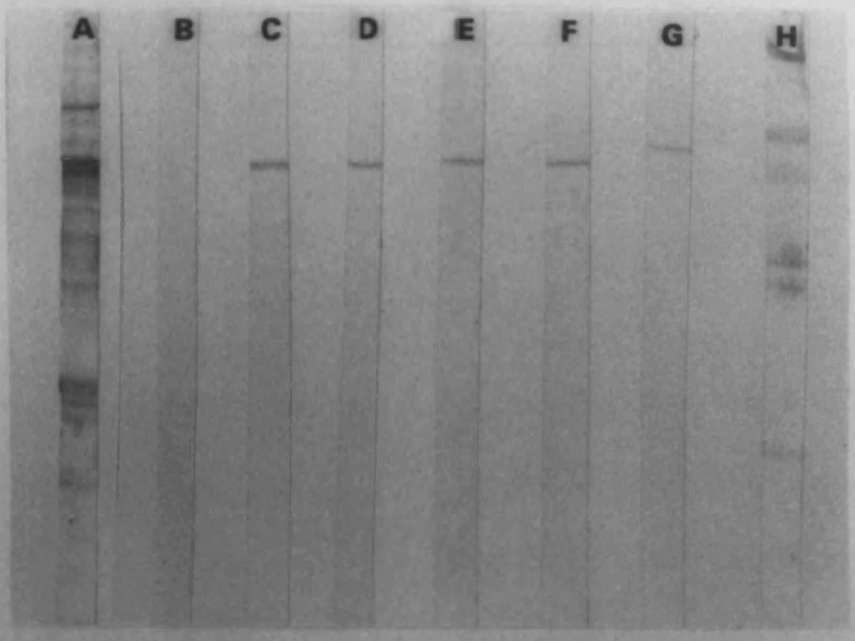


Plate 10 Characterisation of antiserum by Western blotting:

(1) Choice of serum dilution

50 µg nuclear extract per lane was resolved by SDS-PAGE, and transferred to nitrocellulose. Test serum dilutions (serum 82) of 1 in 25, 1 in 50, 1 in 100, 1 in 200 and 1 in 400 (lanes C to G) were used for the primary incubation, followed by development as described in the text.

Lane A shows the blotted protein species stained with Indian ink and lane H the transferred high molecular weight markers. Lane B shows the result of using normal, non-immune rabbit serum, for the primary incubation.

Degradation of purified ADPRT by SDS treatment prior to electrophoresis had already been observed (section 5.3.1) and coupled to the action of endogenous proteolytic factors undoubtedly present in such a crude extract, the observed pattern was not unexpected.

The 116 Kd band was very sharp and faintly visible, even at a 1 in 400 dilution. In terms of optimal colour development and acceptable non-specific background levels, a dilution of 1 in 100 was considered suitable and unless otherwise stated, used in all further studies.

Sensitivity of the method

Assuming the recovery of ADPRT from the purification protocol to be 100%, the equivalent of 2.5 to 25 ng ADPRT in salt extract of nuclei were probed with serum 82. [500 µg affinity purified ADPRT, from a total of 1 g salt extracted nuclear protein, was a typical recovery - the total amount of extract protein run in each gel lane was therefore 5, 10, 20 and 50 µg (Plate 11).]

25 ng, as in the previous experiment, showed a distinct banding pattern with the major component corresponding to 116 Kd. Even 2.5 ng was detected, however, although the band was quite faint and the contamination could not be seen.

If the activity recovered (34%) represented the 'total' ADPRT originating protein, then the serum was capable of detecting 7.5 ng of enzyme. As the substrate incubation period was quite short (30 minutes) and the serum concentration quite low, it was feasible that increasing one or both of these factors would allow detection of smaller quantities of enzyme.

PLATE 11 Characterisation of antiserum by Western blotting:

(2) Sensitivity

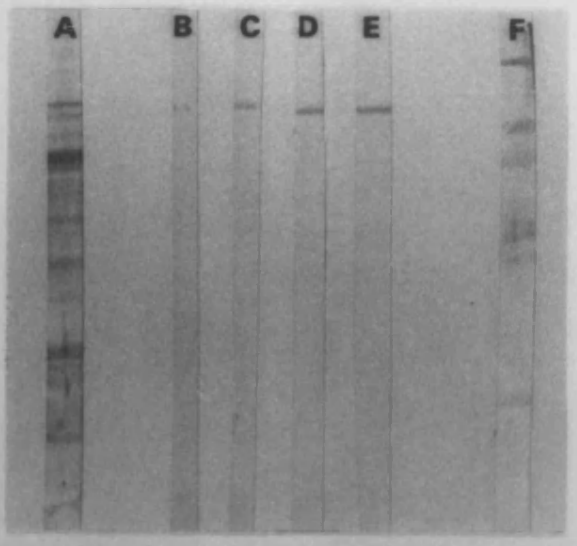


Plate 11 Characterisation of antiserum by Western blotting:

(2) Sensitivity

5, 10, 20 and 50 µg (lanes B to E) of nuclear extract were resolved, transferred to nitrocellulose and probed with a 1 in 100 dilution of serum 82.

Lanes A and F show Indian ink stained blots of nuclear extract and high molecular weight markers respectively.

Comparison of sera 81, 82 and 107

Thus far only serum 82 had been partly characterised and had been shown to bind a 116 Kd protein, as well as a number of smaller proteins to a lesser degree. While it could not be firmly stated that the smaller proteins were fragmented ADPRT, the antigen preparation method did support this.

Serum 82 had been chosen for initial analyses because it had exhibited the highest end point titre by ELISA. The specificity of the other two sera was subsequently tested alongside serum 82. 50 µg nuclear protein was probed as previously with 1 in 100 dilutions of the sera and the developed pattern is shown in Plate 12.

The pattern observed for sera 81 and 82 was very similar, with the same degree of apparent fragmentation. Serum 107 showed one band at 116 Kd, although this was quite faint. A control blot probed with NRS showed no banding.

Proteolysis effects

The immune response from the animals was quite unusual in that all three antisera contained ADPRT specific antibodies. The presence of the other sub-116 Kd bands had to be explained satisfactorily, however. The appearance of a 116 Kd band on a blot probed with antiserum raised to any of the fragments observed would have provided conclusive evidence of physical or enzymatic proteolysis. For obvious practical reasons, this was not feasible however.

Inhibition of proteolytic activity

Two batches of nuclear extract were prepared in parallel, one in the usual way, supplemented with 25 mM sodium bisulphite, and

PLATE 12 Characterisation of antiserum by Western blotting:

(3) Comparison of sera 81, 82 and 107.

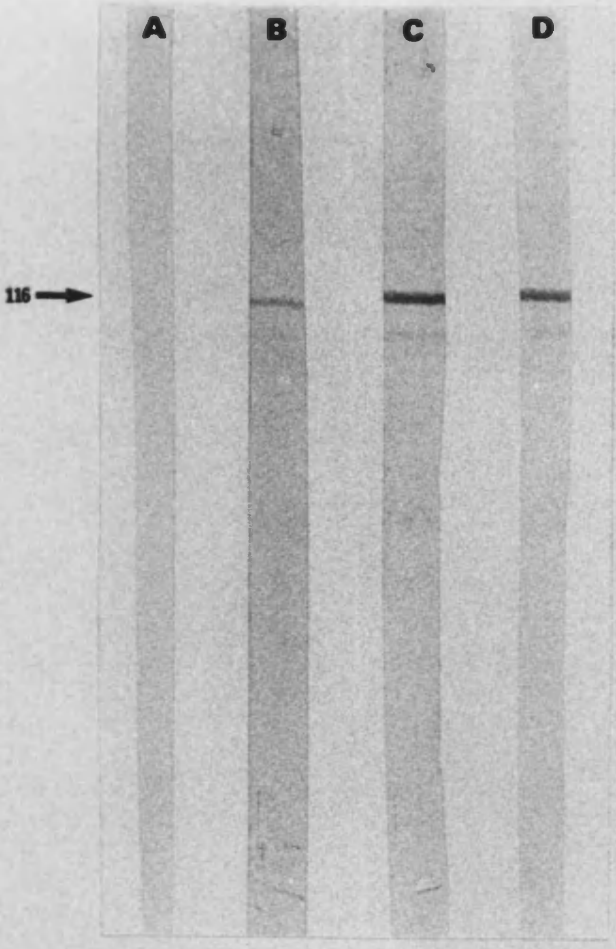


Plate 12 Characterisation of antiserum by Western blotting:

(3) Comparison of sera 81, 82 and 107.

50 µg nuclear extract per lane was resolved, transferred and probed with 1 in 100 dilutions of the three sera. Lane A shows the result for non-immune rabbit serum and lanes B to D for sera 107, 81 and 82 respectively. The position of migration of β-Galactosidase is arrowed.

the other supplemented with bisulphite, plus a protease inhibitor cocktail (0.15 mM phenylmethanesulphonyl fluoride (PMSF), 10 mM EGTA, 5 mM EDTA and 2 mM *p*-aminobenzamidine.HCl.

50 µg of each preparation were blotted and probed as described previously, with a 1 in 100 dilution of serum 82.

In the presence of protease inhibitors (Plate 13, lane C), the large fragments observed previously (lane A arrowed) were reduced to almost invisible levels. In both tracks, however, a diffuse band was visible, approximately 40 to 60 Kd in size.

Preincubation of antiserum with antigen

If the antiserum could be reacted with purified antigen prior to probing the blotted species, specific antibodies would have their ADPRT binding sites occupied and be therefore unavailable for interaction with the immobilised proteins. If the serum was monospecific, the intensity of the banding pattern in the developed blot would be expected to be consistently reduced, by virtue of commonly held epitopes on the fragments and native enzyme. Conversely, if the serum was specific for a variety of proteins, preincubation would result in reduction in intensity of only ADPRT originating species.

60 µg affinity purified ADPRT was mixed with 20 µl undiluted serum 82 and incubated at 4 °C overnight. The antibody/antigen mixture was diluted to 8 ml with PBS-Tween-Casein, and 5 ml used to probe a blot of salt extracted nuclear proteins. Control tracks were probed with 5 ml aliquots of NRS and serum 82 (1 in 400).

The preincubated probe (Plate 14, lane A) showed one faint band at 116 Kd and a very faint diffuse band of intermediate weight as previously. Omission of the preincubation step (lane B) resulted

PLATE 13 Characterisation of antiserum by Western blotting:

(4) Inhibition of proteolysis by inclusion of protease inhibitors.

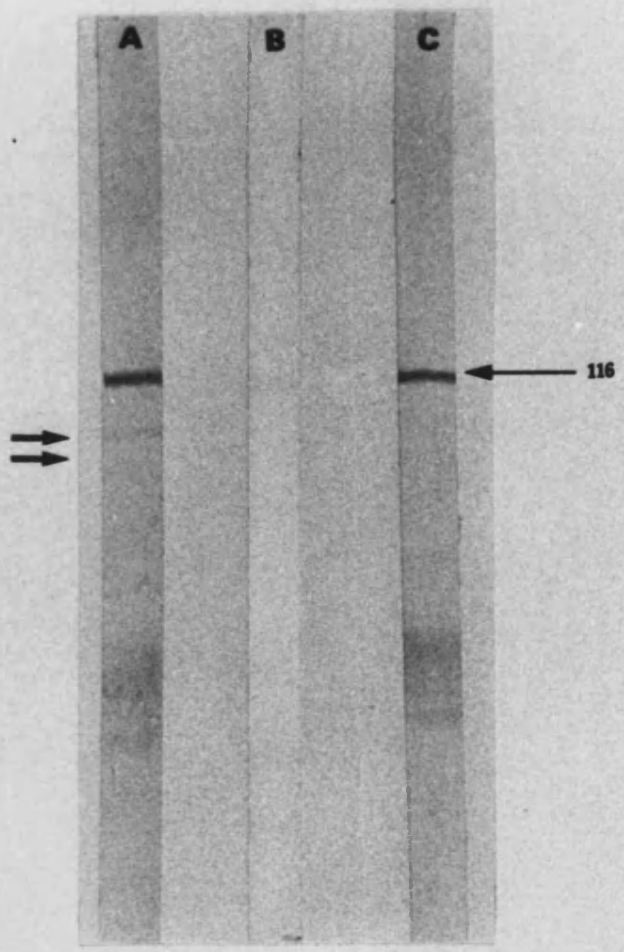


Plate 13 Characterisation of antiserum by Western blotting:

(4) Inhibition of proteolysis by inclusion of protease inhibitors.

50 µg of nuclear extract, isolated in the absence (lane A) and presence (lane C) of protease inhibitors, were resolved, transferred and probed with a 1 in 100 dilution of serum 82 (see text for details).

The arrowed bands in lane A indicate immunoreactive peptides only detected in the absence of protease inhibitors. Lane B shows the non-immune rabbit serum control.

The position of migration of β-Galactosidase is arrowed.

PLATE 14 Characterisation of antiserum by Western blotting:

(5) Pre-incubation of antiserum with affinity purified antigen

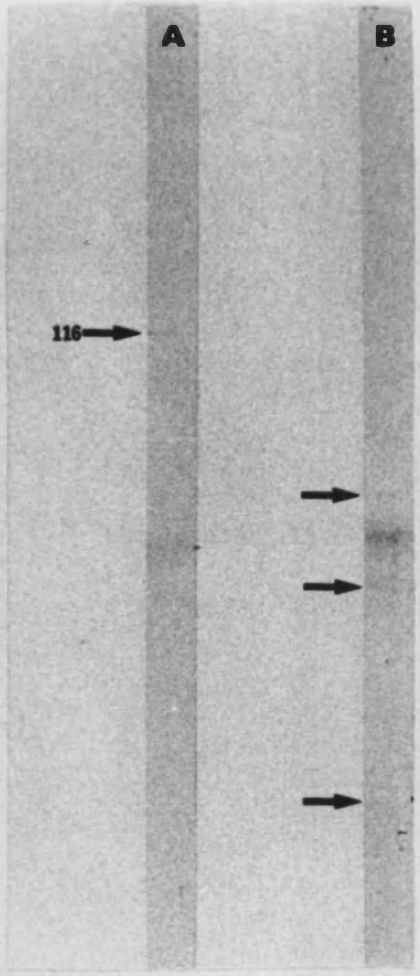


Plate 14 Characterisation of antiserum by Western blotting:

(5) Pre-incubation of antiserum with affinity purified antigen. Two 50 µg samples of nuclear extract were resolved and transferred to nitrocellulose. One lane (B) was probed as previously, except with a 1 in 400 dilution of serum 82, and the other (lane A) with a 1 in 400 dilution of serum 82, which had been previously incubated with affinity purified ADPRT (see text for details).

The arrowed bands in lane B indicate immunoreactive peptides not detected by the pre-incubated serum.

The position of migration of β-Galactosidase is also arrowed.

in the heterogenous banding pattern observed previously. The main, sharper bands are arrowed.

There was no discernible difference in the intensity of the banding at 116 Kd with or without preincubation. As pre-incubation was carried out in the presence of native enzyme, this appears to signify a greater proportion of antibodies specific for the fragments than the intact protein and hence their greater anti-genicity.

Stimulation of proteolysis

If endogenous proteolytic factors were responsible for the banding pattern observed previously, prolonged exposure of the enzyme to such factors at an elevated temperature would be expected to alter the resulting band pattern.

Salt extracted nuclei isolated in the presence of bisulphite only, were incubated for 3 h at 37 °C. Aliquots were taken at 0, 30, 60, 120 and 180 minutes and immediately incubated in SDS-PAGE sample buffer (section 2.4) for 3 minutes at 100 °C. The equivalent of 50 µg of each aliquot was then probed with serum 82 diluted 1 in 100.

The result (not shown due to loss of negative) showed gradual loss of the 116 Kd band to almost negligible levels at 180 minutes. The diffuse band showed partial loss of intensity up to 60 minutes, after which no further change was observed. The higher molecular weight fragments (sub 116 Kd) showed a peak after 30 minutes, which gradually disappeared over the following 90 minutes.

6.3.5 Cross reactivity studies

A preliminary investigation was carried out to determine possible cross reactivity of antiserum with crude extracts of *Trypanosoma brucei* and *Capsicum annuum* plastids. Data from this laboratory and elsewhere (Farzaneh *et al.*, 1985) have strongly suggested a role for ADPRT in trypanosomes. An ADPRT has recently been suggested in mitochondria (Masmoudi and Mandel, 1987), while no data have been published for its presence in plastids.

Assuming conservation of structure, immunochemical studies have distinct advantages in that enzyme activity is not always necessary and that extensive purification of antigen is not required.

6×10^9 *Trypanosoma brucei* were pelleted by centrifugation in a bench centrifuge at 4000 rpm. Two volumes of 50 mM Tris.HCl pH 8.0, 25 mM $Na_2S_2O_5$, 0.5 mM DTT, 0.6 M NaCl were added and the suspension was vortexed prior to sonication, in a sonicating water bath, for 10 minutes at 0 °C. The suspension was then spun at 12000 g for 10 minutes and the supernatant used immediately as crude extract.

Red and green *Capsicum annuum* fruit tissue was homogenised in ice cold buffer A (0.25 M Tris.HCl pH 7.6, 0.25 mM sucrose, 0.01 M $MgCl_2$, 0.04 M β-ME) and filtered through muslin. The filtrate was spun for 10 minutes at 4 °C in a bench centrifuge at 4000 rpm and the pellet resuspended in buffer B (0.05 M Tris.HCl pH 8.0, 0.1 M MgCl , 0.04 M β-ME). The suspension was then layered onto a stepwise sucrose gradient, 20 to 55% w/v, and after centrifugation, the lower plastid band collected. An equal volume of buffer B was added and the suspension centrifuged at 4000 rpm again for 10

minutes. Finally the pellet was resuspended in buffer B supplemented with 5% v/v Triton X-100 and freeze/thawed to liberate protein. why was no salt included in the extraction buffer ?

Approximately 40 µg of each plastid protein extract and 120 µg *Trypanasoma brucei* were blotted alongside 50 µg pig thymus nuclear extract and probed with a 1 in 100 dilution of serum 82 (Plate 15).

The pig thymus protein (lane C) showed one band of 116 Kd with a faint diffuse band as seen previously. The *Trypanosoma brucei* extract (lane B) showed heterogenous banding, mostly greater than 116 Kd in size, although the 116 Kd band was visible and probably the most intense. The diffuse, intermediate size band was also seen. The plastid extracts (lanes D and E) showed only faint banding of intermediate size corresponding to the other extracts. A control *Trypanasoma brucei* track blotted with NRS showed no banding.

Proteolytic degradation of the protein species can be a major reason for concern, as discussed previously, and were further cross reactivity studies to be carried out, a number of prerequisites would appear to be necessary.

(1) The amount of total protein and the dilution of antibody used should be optimised. Heterogenous banding observed in many immunoblots can result from non specific binding due to excess antibody, antigen or both.

(2) Partial purification of antigen prior to separation and blotting reduces the overall amount and variety of species under analysis and can therefore lower non specific interactions.

PLATE 15 Preliminary cross-reactivity studies

where is the arrow

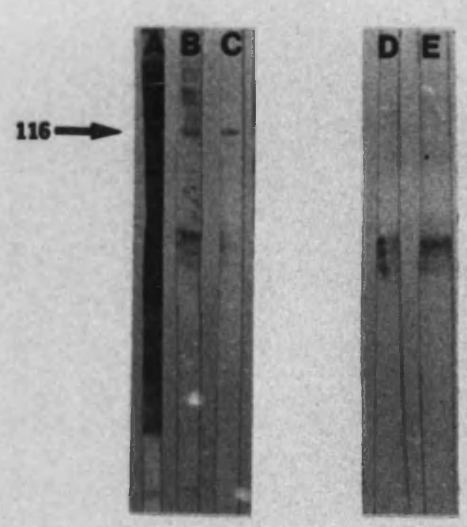


Plate 15 Preliminary cross-reactivity studies.

40 µg of plastid protein extracted from green and red *Capsicum annum* and 120 µg of protein extracted from *Trypanasoma brucei* were resolved alongside 50 µg pig thymus nuclear extract (see text for details), transferred to nitrocellulose and probed with a 1 in 100 dilution of serum 82.

Lane A shows transferred *Trypanasoma brucei* proteins stained with Indian ink. Lanes B to E represent immunoblots of *Trypanasoma brucei*, pig thymus and green and red *Capsicum annum* plastids respectively.

The position of migration of β-Galactosidase is arrowed.

(3) Affinity purification of antibodies using agarose or nitrocellulose immobilised antigen as ligand can prove particularly important. As well as removing the majority of the immunoglobulin and serum protein species and hence lowering non specific interactions, the affinity purified antibodies are known with certainty to be specific, although not necessarily monospecific, for the immobilised antigen.

(4) Where possible, equal amounts of enzymic activity should be analysed. This allows more meaningful quantitative assessment of any structural similarities observed.

(5) Proteolysis should be kept to a minimum by reducing extraction times, using only fresh extracts, using protease inhibitors and using SDS-PAGE sample buffers under conditions of reduced severity.

Blotting of freshly prepared nuclear extract, as opposed to freeze/thawed extract, resulted in a significant reduction in fragmentation, particularly with regard to the intensity of the intermediate size diffuse band.

In terms of cross reactivity of anti pig thymus ADPRT antiserum with the crude extracts examined, the experimental observations suggest that *Trypanosoma brucei* only possesses an ADPRT of similar structure to the pig thumus enzyme. This, however, was only a preliminary study and following optimisation of the system immunoreactive peptides may also be observed in plastids.

6.3.6 Unsuccessful attempts to raise antiserum prior to development of Polymin step

Prior to development of the Polymin step, an attempt was made to raise antiserum in 2 rabbits using affinity purified ADPRT as

antigen. At this stage, the yields of enzyme were very low (typically 5-10% of those subsequently achieved with Polymin) and accordingly only about 10 µg ADPRT was used in the immunisation step. This was injected i.m. in Freunds Complete Adjuvant followed by boosting with similar quantities 28 days later. Test bleeding of the animals was carried out 5 days later and the serum obtained analysed by attempting immune precipitation of enzyme activity and double diffusion in agarose gels.

Reaction of purified enzyme and crude extract with antiserum, followed by precipitation with Donkey-anti-rabbit serum failed to remove any enzyme activity. Double diffusion of antiserum against crude and purified enzyme also gave negative results.

CHAPTER 7

Discussion

The primary objective of the work described in this thesis was to develop a specific means of ADPRT modulation employing antisense mRNA technology. The route to achieving such control was partly realised.

Purification of ADPRT to homogeneity was fundamental to the success of the approach, and while this presented a number of difficulties, an efficient three step procedure was developed which gave consistent purification in the order of 5000 fold.

The nuclei isolation and extraction method, as described by Tsopanakis (1978) was used as an initial enzyme enrichment step. The presence of significant quantities of DNA in the preparation following centrifugation is an undesirable consequence of this method, however, and renders the extract sticky and difficult to process in workable quantities.

Initially, attempts were made to remove this residual DNA by ion exchange chromatography. Murine L1210 DNA, radiolabelled with tritiated thymidine, was used as a marker, and removal observed to be very efficient. The ionic strength dependancy of this method for success, and hence dilution of the extract to suitable salt concentrations, made control of DNA/enzyme reassociation difficult. Loss of large quantities of total enzyme activity through reassociation with resin bound DNA was a recurring problem leading to widely varied yields from preparation to preparation.

The observation that addition of the polycation, PEI, to isolated nuclei prior to salt extraction allowed elution of the chromatin bound proteins while overall nuclear structure was maintained, represented a major developmental step forward. Presumably, extensive electrostatic interaction between the polar head groups of the membrane lipids and the positively charged PEI backbone is responsible for binding the nucleus together. Use of PEI approximately quadrupoled the yield of enzyme extracted at this stage and the DNA present was reduced to almost undetectable levels as measured by ADPRT activating ability. Whereas extraction of nuclei in the absence of PEI necessitated generation of large volumes for DNA removal, treatment of nuclei with PEI had the attraction of allowing further processing of the extract directly by HA chromatography, in addition to the benefit of increased yield. HA chromatography gave consistently good separation and high yields with significant purification ($\leqslant$12 fold).

A number of approaches to affinity chromatography have been utilised in the purification of ADPRT. Okazaki and co-workers (1976) used Sepharose-nicotinamide chromatography as a final step in the purification of calf thymus enzyme, while DNA (Petzold *et al.*, 1981; Agemori *et al.*, 1982) and the azodyes (Zahradka and Ebisuzaki, 1984) have been used with varied degrees of success.

Until 1986, however, and the report of Burtscher and co-workers, the use of the 3-substituted benzamides, ideal candidates for affinity ligands, had not been documented. Attempts were first made to link 3HB to Sepharose 6B *via* a bis-oxirane linker, thus constituting a stable, charge free matrix. The recommended conditions for coupling, however, were considered too harsh for the integrity of the ligand

and attachment under milder conditions was unsuccessful in generating a matrix capable of efficiently binding ADPRT. Whether this lack of interaction was due to inaccessibility of the ligand or low coupling efficiency was not determined.

A modified version of the method of Burtscher and co-workers was therefore adopted which utilises cyanogen bromide activation of Sepharose to attach ligands to the matrix. Binding of enzyme activity by the matrix was observed to be very efficient (>95% in most cases). Unfortunately, however, this presented an unexpected problem initially, as elution of bound activity proved difficult. This was found to be due to over substitution of the matrix and reduction of coupling to 0.3 µmoles of inhibitor (3AB) per ml of Sepharose, using radiolabelled ligand as a marker, subsequently allowed specific elution of enzyme without requiring excessive consumption of competing inhibitor. Elution of batch systems proved possible with lower concentrations of inhibitor than those required for column systems, probably reflecting readsorption to the matrix in the latter case.

Ionic interactions were shown to be a minor constituent of enzyme/ligand binding as attempts at non specific elution by increased ionic strength or pH drop proved only partly successful.

Successful specific elution, on the other hand, proved to be time and free inhibitor concentration dependant. 1 mM 3AB was sufficient to elute enzyme from batch systems, although the elevated concentration of 10 mM 3AB was routinely used to elute column systems in an effort to compensate for the differential elution characteristics of the two approaches.

SDS-PAGE analysis of the affinity purified enzyme consistently showed one major constitutent of approximately 116 Kd and a number of much lesser, smaller constituents. ADPRT had been previously shown to be susceptible to proteolytic degradation during isolation (Jongstra-Bilen *et al.*, 1981; Holtlund *et al.*, 1983) and recently to chemical degradation during boiling prior to SDS-PAGE (Ushiro *et al.*, 1987). The possibility of degradation was therefore not discounted.

Further attempts at removal of contaminating species by gel filtration and hydrophobic affinity chromatography were unsuccessful with analytical SDS-PAGE banding patterns being similar in all cases. The appearance of some of the contaminants following SDS-PAGE of proteins eluted from excised gel pieces added to the lability argument.

Standard characterisation studies such as IEF, total amino acid analysis and NH_2-terminal amino acid analysis were consistent with reports in the literature. The P_i of the enzyme was found to be approximately pH 9.5-10, while no NH_2-terminal amino acid could be observed using the dansylation technique. The amino acid composition was essentially similar to published data with high lysine (12.1%), rather than arginine (3.1%) content, as expected.

Use of SDS-agarose gel electrophoresis to obtain 'intact', pure enzyme, and the use of the entire excised gel slice as immunogen, proved highly successful in generating polyclonal antiserum against the enzyme in rabbits. The sera obtained were characterised by standard immunochemical procedures. Western blotting showed the sera to be specific for a major protein of approximately 116 Kd in pig thymus nuclei salt extract. A number of smaller peptides also

exhibited immunoreactivity. This probably reflected proteolysis, as discussed earlier, although cross reactivity of other DNA and NAD^+ binding proteins could be responsible, a possibility open to further investigation.

Preliminary attempts to immunochemically analyse other tissues with the antisera were largely unsatisfactory. In such studies, more meaningful results are obtained if equivalent amounts of enzyme activity are probed, and similarly isolation of IgG, or preferably affinity purification of specific antibodies, prior to Western blotting, gives more acceptable, less ambiguous data. Such specific antibodies could also be used to synthesise an immunoaffinity adsorbant, allowing rapid purification of further quantities of enzyme.

Unprocessed antiserum proved capable of inhibiting enzyme activity in nuclear extract, suggesting the presence of active site specific antibodies. IgG, or the F_{ab} fraction of IgG, specific for ADPRT, could be used to exploit the highly specific nature of immunochemical interactions and inhibit the enzyme in nuclei of permeabilised cell systems. This offers an alternative means of enzyme inhibition, although the accessibility of chromatin bound proteins, particularly in permeabilised cells, is questionable.

Attempts to acquire sequence data with a view to antisense mRNA studies, were pre-empted by Suzuki and co-workers (1987), who published the NH_2-terminal amino acid sequences of 16 Kd and 40 Kd peptides generated by α-chymotrypsin digestion of human placental ADPRT. They used the enzyme purification method of Burtscher (1986), supplemented with a final HPLC, clean up step. Perhaps significantly, they used a C-8 column to separate the peptide fragments by reversed phase

HPLC, whereas unsuccessful attempts to resolve cyanogen bromide generated fragments, described earlier in this thesis, involved a C-4 column.

Alkhatib and co-workers (1987) had previously been successful in cloning a human ADPRT cDNA from a λgt 11 human hepatoma cDNA library, but had not published sequence data.

Using the peptide amino acid sequence data, Suzuki and co-workers synthesised a 51 base oligonucleotide probe which was successfully used to screen a λgt 11 human placental cDNA library. They obtained 3 clones: λPAP 803, λPAP 802 and λPAP 222 of 1.8, 2.1 and 2.1 kilo bases (Kb) respectively. They sequenced the insert from clone λPAP 803 and furthermore identified a 3.6 Kb mRNA species from HL-60 cells using λPAP 802 and λPAP 803 as probes in Northern blot analysis.

They next used the insert from clone λPAP 802 to screen a human fibroblast cDNA library obtained from size fractionated mRNA (3-4Kb) (Uchida *et al.*, 1987). Two colonies were isolated with cDNA inserts of 3.8 Kb. The insert from one of the two colonies was then subcloned into a suitable vector and sequenced by the chain termination method of Sanger.

Soon after, Smulson's group published a human hepatoma cDNA sequence (Cherney *et al.*, 1987). In agreement with the data of Uchida and co-workers, the sequence was composed of a single 3042 base pair open reading frame and the molecular weight of the protein as predicted from the sequence was proposed to be 113, 135 daltons.

The elucidation of such sequence data, as described above for ADPRT, will hopefully prove to be a vital landmark in gaining an increased understanding of the biological functions of poly ADP-ribosylation. Although it would be naive to expect an instant breakthrough, the use of these data in experimental approaches, such as the one outlined in the introduction to this thesis, will hopefully provide a greater insight into this apparently critical feature of DNA metabolism at its most fundamental level.

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